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# REGULATION OF THE KREBS-HENSELEIT CYCLE IN *SACCHAROMYCES CEREVISIAE*

by



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FACULTY OF GRADUATE STUDIES

Regulation of the Krebs-Henseleit Cycle in *Saccharomyces cerevisiae*.  
Different levels of growth with respect to the different substrates,  
different amounts of the cycle were changed. A single-  
step was added to the cycle and another a continuation of

The undersigned certify that they have read,  
and recommend to the Faculty of Graduate Studies for  
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Henseleit Cycle in *Saccharomyces cerevisiae*' submitted by  
Pak-Yu Chan in partial fulfilment of the requirements for  
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## ABSTRACT

Regulation of arginase activity was studied in *Saccharomyces cerevisiae* 9763 grown in various culture media. Different levels of arginase activity were found when the nitrogenous constituents of the media were changed. L-arginine, when added to the culture medium at a concentration of  $2 \times 10^{-2} M$ , gave the highest enzyme activity, while urea and ammonium at the same concentration gave the lowest levels. Intermediate levels were found when other nitrogenous compounds related to the Krebs-Henseleit cycle were used. From these studies it was concluded that the arginase activity of yeast cultures was finely controlled by the presence of both substrate and end products. These changes were reversible on transfer of the cells to suitable media and time course experiments suggested that repression and derepression of this enzyme was involved.

Further studies of these changes in arginase were made using cell-free extracts which had been subjected to gel filtration. These investigations revealed that the effects of urea and ornithine *in vivo* and *in vitro* were different. For example, it was shown that while urea repressed arginase effectively *in vivo*, it had no influence on enzyme activity *in vitro*. L-ornithine, on the other hand, inhibited the enzyme competitively *in vitro*, but repressed arginase to only a very small extent. In order to examine further the role of urea on arginase activity, cycloheximide was added to the



culture media in the presence of this compound. Within 30 minutes after the introduction of this antibiotic, the specific activity of arginase rose steadily, indicating that repression of the enzyme was terminated by this treatment.

Other enzymes related to the Krebs-Henseleit cycle were studied in cultures either devoid of nitrogen or supplemented with L-arginine or urea. In such cultures, it was found that L-arginine repressed ornithine transcarbamylase. Ornithine transaminase was affected by arginine and urea in the same manner as arginase. Urease was in no case detected under the above culture conditions.

To complement the enzyme studies, levels of free ammonium, urea and amino acids in the culture media and cell-free extracts were determined using an automatic amino acid analyzer. After 5 hours of growth, 30% and 25% of the L-arginine and urea, respectively, present in the culture media, were taken up by the cells. Extracts of cells grown in the basic culture medium contained the lowest level of total amino acids. In contrast, extracts of cells grown on arginine supplemented media contained high levels of amino acids. In urea supplemented cultures, the levels of the free amino acids were again considerably increased. However, urea was not detected in these cell-free extracts. In addition, significant quantities of ammonia accumulated in the medium. The results of these analyses are interpreted on the basis of the previous enzyme studies.

The general properties of yeast arginase were studied in



detail, including reaction velocities, pH optimum, the effects of divalent group activators, inhibition by ornithine, Michaelis-Menten constants in different buffered systems, and molecular weight determination using Sephadex G-200. It was found that the yeast arginase was in many respects similar to the mammalian enzyme but distinct from that of *Neurospora*.

It is concluded that the biosynthesis of arginine and its subsequent breakdown to urea and ornithine in yeast is rigidly controlled by enzyme induction, repression and product inhibition.



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## INTRODUCTION

A. *General Mechanisms for Metabolic Regulation*

During the past three decades, research in genetics and biochemistry, while focused mainly towards an elucidation of basic cell activities, has recently emphasized methods for controlling such activities. From such research, knowledge has been gained regarding not only cell replication in given environments, but also the adaptability of cells to varying environments. In these studies, the elaborate control mechanisms of single-celled organisms, which involve special features of gene action and enzyme specificity, were revealed together with an appreciation of their importance in the evolutionary selection of these species. Of particular importance in this regard would be the selection of the most economical pathway for synthesizing each constituent of the cell. This type of control mechanism, when under extensive study, will rapidly merge with the broad problems of enzyme action and enzyme synthesis. The notion of metabolic control mechanisms can be traced back to Pasteur. However, the first important step towards an understanding of metabolic control mechanisms was the recognition that various substrates in different environments can stimulate microorganisms to form enzymes necessary to attack them. Enzymatic adaptation was not extensively studied until Monod *et al.* (1952) reported on their pioneer work. In these studies an



induction of  $\beta$ -galactosidase (Monod *et al.*, 1951; Monod and Cohn, 1952) demonstrated that the induced enzyme was synthesized *de novo* from amino acids.

The process of induction was originally thought to be restricted to adaptative enzymes, in contrast to the constitutive enzymes of biosynthesis (Karstrom, 1938). However, later success in isolation of constitutive mutants which can produce the same enzyme as the inducible parent clearly indicated that inducibility and constitutivity reflect states of a regulatory apparatus, rather than a fundamental difference in the mechanism of protein synthesis (Pardee *et al.*, 1959). Only recently has it become clear that inducers did not play an essential role in protein synthesis, but participate, along with repressors, in a superimposed regulatory system (Davis, 1961). According to Jacob and Monod (1961), this regulatory system was based on the operon model which was under negative control. For each operon there is a regulatory gene which specifies the structure of a repressor. The repressor is a diffusible molecule which binds to the operator gene and thereby inhibits the transcription of the structural genes of that operon. The identification of the repressors of the phage,  $\lambda$ , (Ptashne, 1967), and of the lac operon of *Escherichia coli* (Gilbert and Müller-Hill, 1966), together with the subsequent partial (Gilbert and Müller-Hill, 1967) and thorough (Riggs and Bourgeois, 1968) purification of the latter have established beyond reasonable doubt that these



repressors are proteins.

Riggs and Bourgeois (1968) reported that the lac repressor protein having a molecular weight of 150,000 can dissociate into subunits having molecular weights between 35,000 and 40,000. In his elegant experiment, Ptashne (1967) was able to show that the  $\lambda$  repressor protein binds to a specific region of DNA, namely the operator region. He further demonstrated that a fraction of the protein in a partially purified  $\lambda$  repressor preparation co-sediments with  $\lambda$  DNA, but not with DNA from a phage hybrid,  $\lambda i^{434}$ , which has an altered immunity (operator) region but is otherwise isogenic with  $\lambda$ . The repressor, therefore, binds only specifically with its corresponding operator. The dissociation constant for this binding was of the order of  $2 \times 10^{-10}$  to  $2 \times 10^{-12} M$  (Gilbert and Müller-Hill, 1966). Many workers suggested the lac repressor is an allosteric protein, but there is no direct evidence to support this contention.

In their studies, Gilbert and Müller-Hill (1967) demonstrated that in the presence of an inducer, thio-isopropyl- $\beta$ -D-galactoside, no binding of lac repressor to DNA could be demonstrated. The repressor obviously underwent conformational changes. Experiments with lac repressor (David and Jacob, 1968), or with  $\lambda$  repressor (Wiesmeyer, 1966) showed that very few molecules were synthesized per genome in each generation. For example, in *E. coli*, only 10 lac repressor molecules (Gilbert and Müller-Hill, 1966) and 20  $\lambda$  repressor molecules (Wiesmeyer, 1966) were



synthesized per cell. These findings, together with the fact that lac repressor is stable and not degraded *in vivo* led Barbour *et al.* (1968) to conclude that the "curious kinetics of depression have their origin in the fact that there are only a small number of molecules of repressor per cell".

In response to environmental changes, a cell may commonly change its activity so that its new metabolism is best suited to the new environment. Such changes are usually best followed by determining whether it is due to the absence of an enzyme or rather to its inhibition or induction. In general these metabolic variations are most easily demonstrated in microorganisms. In many cases, both types of control mechanisms exist in the same organism. Gorini (1958) who studied arginine synthesis in *E. coli*, discovered that in those cultures which received all the arginine required for protein synthesis, the level of arginine-forming enzymes was quite low (repressed) and there was no endogenous synthesis of arginine. In all these cultures which received less arginine than was required, these enzymes were derepressed but were kept from forming more than just that amount of arginine required to make up the amount supplied in the medium. It is clear that both repression and end-product inhibition (a feedback inhibition as described by some writers) are functioning in adjusting the physiological activity of the cell to its environment. Other studies of valine (Umbarger *et al.*;



1957), histidine (Moyed, 1961) and isoleucine (Changeux, 1961) metabolism also led to the conclusion that the end-product of the biosynthetic sequence inhibited what appeared to be the first enzyme in the sequence. From these studies, it is revealed that end-product inhibition is sensitive to slight variations in the level of the substance concerned, while repression is primarily concerned with an economy of protein synthesis.

In 1961, Monod and Jacob proposed the allosteric nature of end-product-sensitive enzymes. According to Monod *et al.* (1963) the allosteric proteins are assumed to possess two, or at least two, stereospecifically different, non-overlapping receptor sites. One of these, the active site, binds the substrate and is responsible for the biological activity of the protein. The other, or allosteric site, is complementary to the structure of another metabolite, the allosteric effector, which it binds specifically and reversibly. This view suggests a mechanism for the competitive interaction sometimes seen without invoking overlapping sites, and the relationship between regulatory and substrate molecules which bears no steric resemblance to one another.

#### *B. Regulation of Enzymes Related to Arginine Synthesis.*

The biosynthesis of arginine from ornithine, and the breakdown of arginine by arginase to ornithine and urea was originally proposed by Krebs and Henseleit (1932) to occur via a cycle of reactions in mammalian liver. This has since



been substantiated in a wide variety of organisms including higher plants, (Kasting and Delwiche, 1957), bacteria, (Vogel, 1953) fungi (Vogel and Bonner, 1954) and mammals (Mohamed and Greenberg, 1945).

In yeast, the above studies provide convincing evidence that arginine is synthesized from glutamic acid via ornithine, and Vavra and Johnson (1956) showed that exogenous ornithine, citrulline, and arginine all reduced the amount of glutamate carbon incorporated into arginine and proline. However, an elucidation of the enzymes required for arginine synthesis in yeast was at that time lacking. Subsequently some evidence was obtained which showed that the formation of ornithine in yeast follows a pathway somewhat resembling that of *E. coli* (Vogel and Vogel, 1963), but essentially different from that existing in mammalian systems (Meister, 1965). Reactions resulting in the biosynthesis of ornithine in *Saccharomyces* are summarized in Figure 1. DeDeken (1962) has obtained evidence that yeast preparations can catalyze the reduction of N- $\alpha$ -acetylglutamate to the corresponding semi-aldehyde. Arginine was found to inhibit this reaction, indicating a feedback mechanism of inhibition. The isolation of an enzyme from *Saccharomyces cerevisiae* which can transfer the acetyl groups of N- $\alpha$ -acetylornithine to the amino group of glutamate (DeDeken, 1963) led to the conclusion that the yeast pathway may be more correctly depicted as in Figure 2.

The other key reaction leading to the formation of arginine is the conversion of ornithine to citrulline by the



FIGURE 1. Biosynthesis and Breakdown of Arginine in *Saccharomyces cerevisiae*.

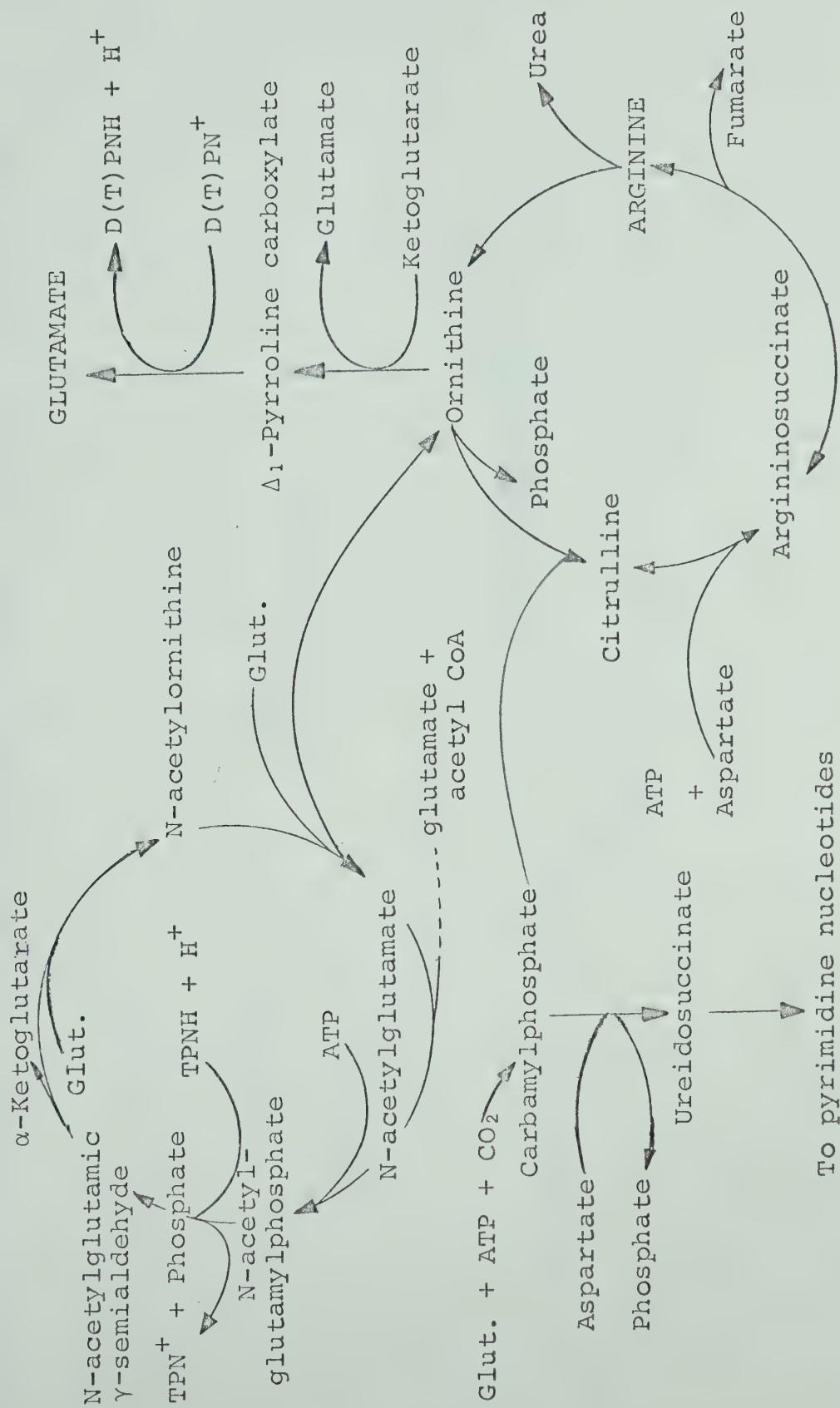
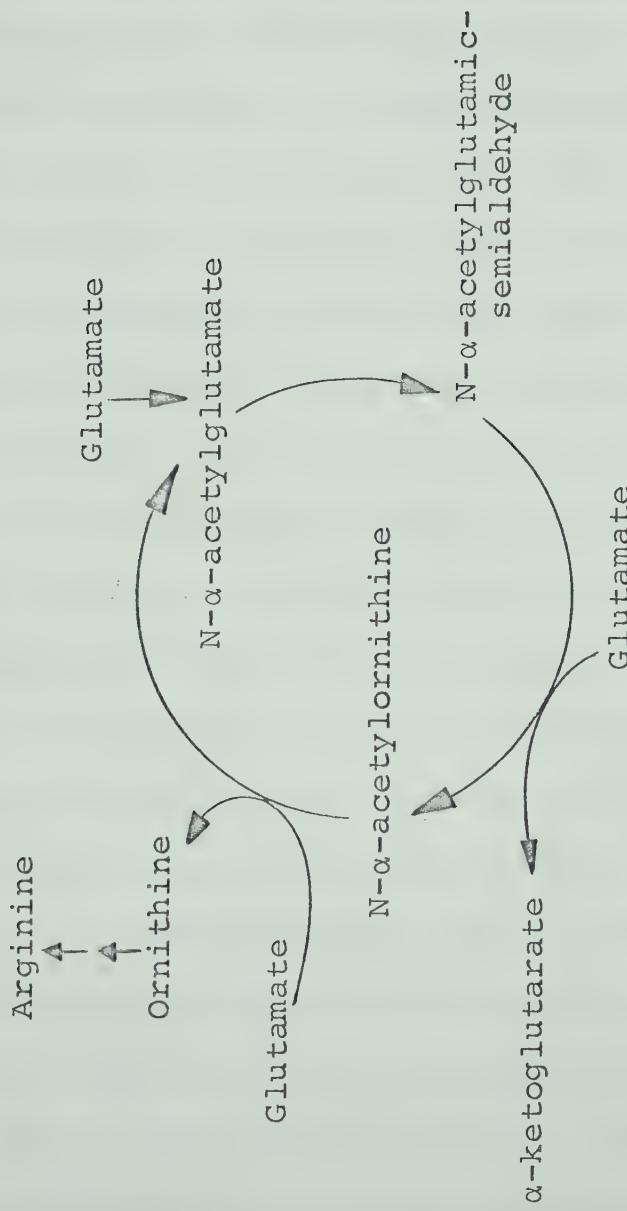




FIGURE 2. Proposed Scheme for Ornithine Synthesis in Yeast (DeDeken, 1963).





enzyme ornithine transcarbamylase (OTC) (EC 2.1.3.3). As in *E. coli*, this enzyme in yeast is strongly repressed by arginine (Bechet *et al.*, 1962). Therefore, two of the steps in the biosynthesis of arginine are repressible by arginine, a fact which would tend to implicate N- $\alpha$ -acetyl intermediates in the biosynthesis of arginine in yeast. Later work by Middelhoven (1969) confirmed the earlier finding that OTC was strongly repressed by exogenous arginine. However, he reported that two other enzymes of the arginine pathway, argininosuccinate lyase (ASL) (EC 4.3.2.1) and acetyl-ornithine-glutamate transacetylase (AGT)\* were not repressed by exogenous arginine. This suggests that the physiological function of the endogenous repression of these two enzymes by arginine is not primarily in the regulation of arginine synthesis but in that of enzyme synthesis itself. Further evidence for this suggestion is supported by the observation that these two enzymes are not derepressed when yeast, growing on peptone as a nitrogen source, is transferred to a mineral salts medium (Middelhoven, 1969).

It is interesting to note that a different control mechanism exists in *Chlamydomonas reinhardtii*. Strijkert and Sussenbach (1969), in their work with this alga, found that the pathway of arginine synthesis was essentially the same as in some bacterial and yeast species namely in having a transacetylation reaction between glutamate and N-acetyl-L-

---

\* Not yet registered by the Enzyme Commission. Middelhoven, 1969.



ornithine and by displaying a feedback control of acetylglutamate kinase by arginine. However, in their report, they mentioned that in the wild type strain, no control of the other enzymes of the arginine pathway occurred except for the feedback inhibition of acetylglutamate kinase. On the other hand, arginine requiring mutants *argC1* and *argG2* behaved quite differently. The former had an argininosuccinate lyase which was effectively derepressed by the addition of 100  $\mu$ g/ml ornithine to the culture medium while this enzyme from the latter mutant was strongly repressed when grown on an arginine (20  $\mu$ g/ml) medium. Compared to argininosuccinate lyase all of the other enzymes in the pathway had little or no regulation of their synthesis. The physiological significance of this regulatory mechanism is, therefore, uncertain as it did not exist in the wild type strain. This is clearly in contrast to the arginine biosynthetic pathway of *E. coli* which contains several enzymes known to be repressed in parallel and which are pleiotropically controlled.

A rather unique regulatory mechanism affecting arginine biosynthesis in yeast has been described by Bechet and Wiame (1965). They added nystatin to the cell suspension to obtain "permeabilization" of the yeast cells. In these "permeabilized" cells, they noted an abrupt drop in ornithine transcarbamylase activity following the addition of arginine to the medium. The activity was reduced to 50% of initial levels after about 15 minutes and further to 10% after 60



minutes. This rapid decline in activity was suggestive of enzyme destruction. Furthermore, it could be prevented by the administration of cycloheximide to the "permeabilized" cells. As cycloheximide is an antibiotic inhibiting protein synthesis (Siegel and Sisler, 1964), this indicates that the inactivation process involved formation of protein. By heating the cells to 59°C, reactivation of the enzyme was observed. Hence, they suggested that under the conditions of repression, arginine induced a synthesis of specific ornithine transcarbamylase binding protein which was heat labile and which resulted in loss of activity *in vivo* after a very limited period of growth. In a later experiment, Messenguy and Wiame (1969) were able to isolate this regulatory protein by column chromatography using Sephadex G-200 and studies of it *in vitro* were conducted. They showed that this regulatory protein had the same molecular weight as arginase. Arginase and OTCase did not form a complex in the absence of two effectors, ornithine and arginine. Thus ornithine, in addition to being a substrate for OTCase, is also an effector of this system and arginine, while being an inducer of the regulatory protein, is also an inhibitor of OTCase under these conditions. It is interesting to note that the  $r^+$  to  $r^-$  mutation in this organism, leading to non-repressibility of OTCase, was accompanied by failure of arginine to induce production of the binding protein. The term "epiprotein" was proposed by Messenguy and Wiame (1969) to designate the regulatory protein. The regulatory arginase, forming an



allosteric system by reversible binding with OTCase, differs from the usual arginase and was termed "epiarginase".

The above findings suggest that the regulation of OTCase is more important than that of other enzymes of the arginine synthesis pathway. The reason for this may be in the fact that ornithine is a precursor of both arginine and putrescine in *Saccharomyces* (Middelhoven, 1969). Putrescine is formed from ornithine by decarboxylation and is the precursor of spermidine, a base universally associated with ribosomes (Tabor *et al.*, 1961). Both putrescine and spermidine have been detected in *Saccharomyces* (Fischer and Bohn, 1957). In divergent biosynthetic pathways the first enzyme after the branching point is generally regulated more precisely than the other enzymes, most commonly by both enzyme repression and by end-product inhibition (Umbarger, 1963). If in yeast, arginine and putrescine are synthesized by divergent pathways, as in *E. coli*, OTCase will be the first enzyme of the arginine-synthesizing branch. This position in the metabolic pathway may, therefore, explain the double regulation of its activity, involving both repression and the action of a specific regulatory protein.

The situation will, however, be far more complicated than the above example if we consider the participation of other metabolic products in control systems. In their work with a wild strain of *E. coli*, Strain B, which produces low levels of arginine synthesizing enzymes, Gorini *et al.* (1961) revealed an antagonism between glucose and arginine in



controlling the levels of these enzymes. A repressive effect by glucose, similar to the widely known "glucose effect", was demonstrated in Strain B. This effect can be counteracted by arginine, which behaves formally as an inducer. Hence, two repression systems control synthesis of these enzymes. Firstly, control by an end-product (arginine), and secondly, control by a product formed in general metabolism. The effect of these two controls appears to be additive.

It is not uncommon that enzymes are capable of existing in different forms which have different properties. For example, when *Bacillus licheniformis* was grown on glucose and L-arginine, Bernlohr (1966) detected two chromatographically distinct types of ornithine transcarbamylase. One enzyme, presumably, had a biosynthetic function and was repressed by arginine. The other was induced by arginine, repressed by glucose, was relatively heat-stable, and was synthesized towards the end of the growth cycle. A similar situation was found to exist in a strain of *Pseudomonas fluorescens* (IRC-204), by Ramos *et al.* (1966 a & b). They also reported the discovery of two ornithine transcarbamylases. One appeared to be catabolic in nature and was induced by arginine, the other enzyme appeared to be anabolic and was repressed by arginine. They were not only different functionally but also physically since the catabolic enzyme had the optimum pH of 6.7 while the pH optimum for the anabolic enzyme was 8.5. In *Neurospora crassa* a completely



different situation was described. Nazario (1967) found that in a mutant of *Neurospora crassa* (arg10), the ornithine transcarbamylase levels were progressively repressed by arginine. However, the mutant failed to form argininosuccinate lyase, and thus accumulated argininosuccinate when grown on an arginine-containing medium. If, however, the argininosuccinate level in the medium was indirectly increased by the addition of citrulline, ornithine transcarbamylase was derepressed. The mechanism of derepression was found to be via the competitive inhibition of arginyl transfer RNA synthetase by argininosuccinate. Earlier, Bock *et al.* (1966) reported that in order to achieve repression of the arginine biosynthetic enzymes, arginyl-t-RNA formation is necessary. When arginyl-t-RNA synthetase is inhibited by argininosuccinate, then the arginine biosynthetic enzymes are derepressed.

### C. The Regulation of Enzymes Related to Arginine Catabolism

The control of arginine catabolism has been studied somewhat less than biosynthesis of this amino acid. Concerning the breakdown of arginine, Edlbacher and Baur (1938) reported the presence of arginase (reaction 1). However, Roche and Lacombe (1952) reported the presence of arginine deaminase (L-arginine:iminohydrolast, EC 3.5.3.6) in baker's yeast. The reaction (2) produced citrulline and ammonia at pH 5.8 to 7.0. The enzyme was readily activated by  $\text{Co}^{++}$ .





pH 8.5 - 10



pH 5.8 - 7.0

As in the case of the arginase from *E. coli*, the yeast enzyme was induced by its substrate (Middelhoven, 1964). It is also now well known that the addition of  $\text{NH}_4^+$  prevents or lessens the induction of arginase under these conditions (Middelhoven, 1968). Whether a competition between  $\text{NH}_4^+$  and arginine for the aporepressor is responsible for this effect or whether a separate repression by  $\text{NH}_4^+$  exists, cannot as yet be decided. Inhibition of arginase synthesis by  $\text{NH}_4^+$  suggests that one of the biological functions of arginine catabolism in yeast is to provide  $\text{NH}_4^+$ . If  $\text{NH}_4^+$  are available in the medium, arginase will no longer provide a precursor of them.

Eliasson and Strecker (1966) have reported experiments on the control of arginase levels in tissue cultures of Changs liver cells. They found that arginase was repressed by proline or  $\Delta'$ -pyroline-5-carboxylic acid, an end-product of ornithine catabolism. They postulated that because ornithine transcarbamylase and ornithine- $\delta$ -transaminase competed for available ornithine, an initial increase in the level of ornithine transcarbamylase in the cells would



lead to a decrease in the level of proline formed. This situation would thus lead to a derepression of arginase synthesis. Since the enzymes of arginine synthesis are known to be repressed by arginine, the ensuing increase in arginase activity would be expected to remove the repressor of the arginine synthesizing pathway.

The studies outlined in this Introduction have helped to provide a much clearer picture of the ornithine cycle in a variety of microbial species. It is clear from these studies that several distinct regulatory mechanisms can be recognized. Each mechanism is well suited to the physiology and metabolism of the organism. Diversity in control mechanisms may not necessarily indicate independent phylogenetic relationships, since, in the course of evolution, natural selection has forced individual species to modify their biological activities to such an extent that different strains may be produced from a single species situated in different environments. Alternatively, a common control mechanism for the regulation of a particular metabolic pathway may be found in various species which have adapted themselves to a common environment. Therefore, in justifying a simple evolutionary origin for a complex metabolic pathway, it is not sufficient to demonstrate common catalytic steps or common control characteristics that regulate these enzymatic reactions (Datta, 1969). Regulatory control is clearly only the step required to ensure balanced production



of the end-products. Hence, it is not surprising that even within a single biochemical pathway, considerable variation in the overall control pattern is the rule rather than the exception.

#### D. The Present Studies

The information outlined above has given a much clearer picture of the ornithine cycle in a variety of plants and microbial species than existed a decade ago. In order that our understanding of the ornithine cycle in yeast, particularly its catabolic aspects, could be enlarged, the present studies emphasized the following major areas.

##### (a) The roles of arginine and urea in the metabolism of *Saccharomyces cerevisiae*.

The importance of ornithine cycle amino acids and urea as nitrogen sources in plants and microorganisms is now well documented (Arnow *et al.*, 1953; Baker and Thompson, 1962; Taylor and May, 1967; and Splittstoesser, 1969). Arginine, being particularly rich in nitrogen, can be recycled to form other nitrogenous compounds. For example, Jones and Boulter (1968) reported that during germination of *Vicia faba* arginine breakdown accounts for 19% of the total nitrogen that is re-incorporated by the tissues. Earlier, Boulter and Barber (1963) examined the levels of various amino acids in germinated and ungerminated seeds of this species. They found that in the ungerminated seeds, arginine was by far the most prominent amino acid, accounting for 25% of the total



amino nitrogen. After 14 days of growth, however, less than 6% of this amount remained but the levels of all other amino acids had increased, indicating incorporation of arginine nitrogen into other compounds.

The utilization of various nitrogen sources by micro-organisms has been known for sometime. For example, Hattori (1958) reported the ability of green algae to assimilate a variety of compounds including ammonia, nitrate, urea, arginine, ornithine and citrulline, presumably at the expense of energy liberated by respiration as this latter process was commonly enhanced on addition of the nitrogenous compounds.

The role of urea in metabolic recycling of nitrogen poses an important question in plants and microorganisms. In animals, the ornithine cycle serves to remove toxic, unwanted ammonia in the form of excretable urea (Forster, 1954; Mora *et al.*, 1965; and Calisky *et al.*, 1968). However, in plants and microorganisms it is to be expected that urea would be utilized in nitrogen metabolism. This subject has been a matter of considerable controversy (Bolland, 1959; Hattori, 1960; Baker and Thompson, 1962; and Hodson and Thompson, 1969), and remains at present a largely unsolved problem. In the present studies it was, therefore, of some interest to examine the ability of yeast cells to utilize arginine and urea as sole sources of nitrogen and to compare the effect of such conditions on the rates of growth and the free amino acid levels.



(b) *Regulation of ornithine cycle enzymes under different culture conditions.*

As outlined above, even a simple unicellular organism is able to develop elaborate control mechanisms to adapt itself to varying environments. Until recently, however, despite intensive work on the mechanisms for control of amino acid metabolism in yeast (Spiegelman, 1951; Ratner, 1955; and Moat and Ahmad, 1965), several aspects of this important subject remained to be examined at the cellular level. For example, the importance of arginine in the regulation of its biosynthesis and hydrolysis can be appreciated by the following work undertaken by different research groups on: (i) feedback inhibition of the N- $\alpha$ -acetylglutamate reductase (DeDeken, 1962); (ii) repression of some enzymes of the biosynthetic pathway (Bechet *et al.*, 1962); (iii) inhibition of ornithine transcarbamylase (Bechet and Wiame, 1965); and (iv) induction of arginase (Middelhoven, 1964). However, apart from the work of Middelhoven (1964, 1969), little detailed work has been published on the regulation of arginase levels in *Saccharomyces* although it is to be expected that the levels of this enzyme would be fairly rigidly controlled. Furthermore, as mentioned above, the fate of urea produced in this reaction and its possible effect on the enzymes of the cycle remains to be investigated.

The pathways involved in urea utilization have been the subject of speculation since certain evidence (Baker and Thompson, 1968; Roon and Levenberg, 1968) indicated that the



reactions used by some organisms might not be universal. On the basis of detailed work, Kasting and Delwiche (1957) reported the occurrence of arginase and urease activities in watermelon seedlings. They concluded that these enzymes functioned in a manner analogous to those of animals. Bollard (1959) has published data which suggests that the assimilation of urea in urease-free higher plants is accomplished through a process which is essentially a reversal of the ornithine cycle. A similar suggestion came from the studies of Walker (1952) with *Chlorella*. However, more recently, Hattori (1960) has postulated that an acceptor, A, in *Chlorella* cells, reacts with either free NH<sub>3</sub> or urea as depicted in reactions 3 and 4.



Kating (1962), using yeast, obtained evidence for a cleavage of urea to a carbamyl residue and ammonia. Valentine and Wolfe (1961) have shown that an extract of *Streptococcus allantoicus* produced glyoxyl-urea when incubated with urea. The glyoxyl-urea was then cleaved phosphorolytically in the presence of diphosphopyridine nucleotide (DPN), magnesium and phosphate, to carbamyl phosphate. Recently, Roon and Levenberg (1969) in their experiments with green algae and yeast, reported a new enzyme catalyzing urea cleavage by a reaction which was adenosine triphosphate dependent. A recent publication indicates that this enzyme occurs also in



*Saccharomyces* when grown in the presence of urea (Whitney and Cooper, 1970).

In the present studies, attempts were made to elucidate the importance of arginine and urea in controlling the activities of certain key enzymes of the ornithine cycle. In such investigations, it was expected that a close relationship would exist between the levels of arginase and other enzymes of the cycle. Considerable emphasis was given to an examination of the influence of exogenous arginine and urea on arginase since these compounds are the substrate and product respectively of this enzyme.

(c) *The general properties of yeast arginase.*

Regulatory mechanisms in microorganism involve changes either in enzyme activity or in enzyme synthesis (Jacob and Monod, 1961; Mora *et al.*, 1965). To understand the properties of the enzyme itself, is, therefore, as important as examining changes in synthesis since both play a parallel role in adjusting the cell's physiological condition. It is not uncommon that the same type of enzyme from different species may behave quite differently. For example, the arginase from uricotelic animals and *Neurospora crassa* is able to hydrolyze exogenous but not endogenously formed arginine (Brodsky *et al.*, 1965; Mora *et al.*, 1966; and Carlisky *et al.*, 1967). These workers also reported that the arginase from the last two organisms is a different protein from that of ureotelic animals. Therefore, identification of *Saccharomyces cerevisiae* arginase as identical with or



different from other species, and knowledge of its properties as well as its relationship with other ornithine cycle enzymes, would be required for an elucidation of the importance and position of this enzyme in this organism.



## MATERIALS AND METHODS

*Chemicals*

L-citrulline and the barium salt of argininosuccinic acid were obtained from Calbiochem, California, U.S.A. L-arginine-HCl, L-arginine (free base), and all other amino acids were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A. Urea (ultra pure) was obtained from Mann Research Laboratories, Inc., New York, U.S.A. Sephadex gels and dextran blue were obtained from Pharmacia Canada Ltd., Montreal, Canada. Cycloheximide, urease solution (80 units/ml) and bovine liver arginase were purchased from Sigma Chemical Company, Missouri, U.S.A. Hycel urea nitrogen reagent was obtained from Hycel, Inc., Texas, U.S.A. L-arginine (guanido-<sup>14</sup>C) was obtained from New England Nuclear, Massachusetts, U.S.A., and scintillation grade 2,5-diphenyloxazole (PPO) was supplied by Nuclear-Chicago, Des Plaines, Illinois, U.S.A. Bactowort Agar was routinely supplied by Difco Laboratories, Detroit, Michigan, U.S.A. All other chemicals were obtained from Fisher Scientific Company, Edmonton. In every instance the chemicals used were of the highest purity available.

*Organism and Methods of Culture*

*Saccharomyces cerevisiae* (ATCC 9763) was purchased from American Type Culture Collection, Rockville, Maryland, U.S.A. and maintained in culture as described below.



*Preparation of Agar Slant*

25 gm of the bacto-wort agar were suspended in 500 ml of demineralized water and steamed for 10 minutes with constant stirring. 10 ml of the resulting solution were then pipetted into 20 x 150 mm test-tubes which were plugged with absorbent cotton, and autoclaved for 10 minutes at 15 psi. The tubes were tilted to form slants and allowed to cool. After an incubation period of 24 hours at 30°C, any slants which showed growth were discarded. The remainder were stored in the refrigerator at 4°C for subsequent inoculation.

*Maintenance of Stock Cultures*

Yeast cells were routinely maintained by transferring the cells to new agar slants every 2 weeks. Such transfers were incubated at 30°C for 2 to 3 days and followed by storage in the refrigerator at 4°C.

*Preparation of inoculum*

To prepare an inoculum, yeast cells were transferred from the solid medium to a liquid stock medium. This latter medium consisted of 100 parts of a basic culture medium and 1 part of an amino acid solution. A small loop of yeast cells was added to 500 ml of this stock culture medium contained in a 1000 ml Buchner flask. The side arm was attached to a vacuum system. The culture was aerated by drawing sterile air through the medium. Asceptic technique was employed throughout. Such liquid cultures were



incubated for 24 or 36 hours as indicated at 30°C. Cells, which were at this stage in the exponential growth, were then harvested by centrifugation at 4000 x g for 10 minutes in a refrigerated centrifuge (International Equipment Company, Massachusetts, U.S.A., Model B-20). The supernatant liquid was retained in cases where analyses for arginine and ammonia were conducted, otherwise it was discarded. The cells were washed (3 times) with cold sterile deionized water and resuspended in 3 - 5 ml of ice-cold, sterile distilled water.

*Conditions for growth of experimental cultures*

Aliquots of the above dense cell suspension were added to Buchner flasks containing appropriate concentrations of various nitrogen sources added as supplements to the basic culture medium in different experiments. The cells were incubated at 30°C (pH 4.5) for a time period appropriate to the particular experiment and with vigorous aeration. The cells were then harvested in the exponential growth phase after chilling the culture in ice for 5 minutes.

*Preparation of liquid culture medium*

(a) *Basic culture medium.*

This liquid culture medium, although completely devoided of any nitrogen source, contained sufficient carbon, vitamins and mineral salts to support growth of the yeast cultures.

Table 1 summarizes the composition of this medium.



TABLE I

## CONSTITUENTS OF THE BASIC CULTURE MEDIUM

Constituents	Amount/litre
Dextrose	100 g
Vitamins	
Thiamine	0.5 mg
Inositol	50 mg
Biotin	0.02 mg
Calcium pantothenate	2.5 mg
Nicotinic acid	2.5 mg
Mineral salts	
KCl	0.85 g
MgSO <sub>4</sub> · 7H <sub>2</sub> O	0.515 g
FeCl <sub>3</sub> · 6H <sub>2</sub> O	5 mg
MnSO <sub>4</sub> · H <sub>2</sub> O	5 mg
CaCl <sub>2</sub> · 2H <sub>2</sub> O	0.25 g
KH <sub>2</sub> PO <sub>4</sub>	1.1 g
Organic acids	
Potassium citrate	10 g
Citric acid	2 g



*Preparation of stock solutions:*

All stock solutions were stored in the refrigerator at 4°C prior to use. They contained the following components and were prepared as indicated.

- (i) Citrate buffer solution: 100 gm potassium citrate and 20 gm citric acid were dissolved and made to 1 litre with deionized water.
- (ii) Vitamin solution I: 10 mg thiamine and 1 gm inositol were dissolved in 200 ml deionized water and was made up to 1 litre.
- (iii) Vitamin solution II: 10 mg biotin were dissolved in 100 ml 50% ethanol solution (ethanol:water, 1:1 v/v). 200 mg calcium pantothenate and 200 mg nicotinic acid were dissolved in about 200 ml water; 8 ml of the above biotin solution were added and the volume was brought up to 1 litre with deionized water.
- (iv) Salt solution I: 17 gm KCl, 10.3 gm  $MgSO_4 \cdot 7H_2O$ , 100 mg  $FeCl_3 \cdot 6H_2O$ , and 100 mg  $MnSO_4 \cdot H_2O$  were together dissolved in about 800 ml distilled water. 2 ml of concentrate HCl were added. 5 gm  $CaCl_2 \cdot 2H_2O$  were dissolved in 100 ml water. This was added to the first solution and the whole made to 1 litre with deionized water.
- (v) Salt solution II: 22 gm of  $KH_2PO_4$  were dissolved in deionized water and made to 1 litre.

To make 1 litre of basic culture medium, 100 ml citrate buffer, 50 ml vitamin solution I, 25 ml vitamin solution II,



50 ml each salts solution I and II were added to about 500 ml deionized water. 100 g dextrose were dissolved in this solution. The medium was then adjusted to pH 4.5 by addition of 5 M citric acid, and the final volume was then made to 1 litre with deionized water.

(a) *Preparation of amino acid stock solutions*

Table II gives the levels of the different amino acids present in the amino acid stock solution. The amino acids were dissolved in 50 ml of deionized water at 50°C. Complete solution was achieved by the addition of 10 ml concentrated HCl. The final volume was made to 250 ml with deionized water.

All culture media were adjusted to pH 4.5, sterilized in the autoclave for 10 minutes at 15 psi, cooled and inoculated within 24 hours of preparation.

*Measurement of cell growth*

(a) *Determination of the growth curve.*

The growth of yeast cells in different culture media was followed spectrophotometrically using Beckman DB-G spectrophotometer. Optical density at 500 nm was measured frequently throughout the growth period.

(b) *Determination of dry weights.*

After harvesting and washing with distilled water, an aliquot of the cells was suspended in 2 - 3 ml of distilled water in a 50 ml plastic centrifuge tube. The suspension



TABLE II

## COMPOSITION OF THE AMINO ACID STOCK SOLUTION

Amino acid	g/250 ml of solution
DL-leucine	1.0
L-isoleucine	0.5
DL-valine	1.0
L-cystine	1.0
L-tryptophane	1.0
L-tyrosine	1.0
DL-phenylalanine	1.0
L-glutamic acid	2.0
DL-threonine	1.0
DL-alanine	1.0
L-aspartic	1.0
L-lysine HCl	1.0
DL-methionine	1.0
L-histidine HCl·H <sub>2</sub> O	0.5
DL-serine	1.0
L-proline	0.25
Glycine	0.50



was frozen by immersion in a dry ice-acetone bath. The frozen cells were then lyophilized in a Virtis Automatic Freezer-Dryer (Model 10-010) for 15 hours or until a fine powder had formed.

#### *Sephadex column chromatography*

Sephadex G-25 was suspended in distilled water and boiled for 1 hour with constant stirring. The fines were removed by decantation and the gel was then placed *in vacuo* to remove air bubbles and followed by pouring into a jacketed column under gravity to give a final bed of 1.6x 20 cm. The Sephadex was washed with 200 ml of 0.01 M potassium phosphate buffer (pH 7.5) and kept at 4°C until required.

Sephadex G-200 was soaked in 0.01 M potassium phosphate buffer (pH 7.5) for 3 days at 4°C with occasional stirring. The fines were removed by decantation, air was removed *in vacuo* and the gel was packed into a jacketed column giving a final bed volume of 2.8x51 cm. The Sephadex G-200 column was then kept at 4°C until required.

#### *Cell-free extracts*

At the end of the culture period, the cells were harvested and washed three times with ice-cold distilled water. The cells were resuspended in 10 mM 2-mercaptoethanol containing 1% NaHCO<sub>3</sub> (Middelhoven, 1964), 0.5 ml being used per g.f.wt. of cells. The cells were disintegrated by treatment in a Fisher Ultrasonic Generator (manufactured by



Blackstone Ultrasonic Inc., Model SS 0). The suspension, contained in a 50 ml plastic centrifuge tube, was immersed in an ice bath. In every instance, the cells were subjected to three 1 minute periods of sonication with pauses of 60 seconds between treatments to prevent excessive heating. The suspension was then diluted 4-fold by addition of 0.01 M potassium phosphate (pH 7.5). Cellular debris were removed by centrifugation for 10 minutes at 18,000 x g. The supernatant was stored at 4°C. No loss of arginase activity was observed when extracts were stored at 2-4°C for periods up to 5 days. Losses of 50-60% of arginase activity occurred after storage periods of 10 days.

*Partial purification of cell-free extracts*

(a) *Sephadex G-25 treatment.*

All procedures were carried out at 4°C. The cell-free extract (3 ml), containing approximately 25-35 mg protein, were applied to a 1.6x20 cm column of Sephadex G-25 and eluted with 0.01 M potassium phosphate (pH 7.5). Fractions of 3 ml were collected at a flow rate of 60 ml/hr using a refrigerated Buchler Fraction Collector (Model 3R-4000). The bulk of the applied protein was collected in fraction 6-8. These fractions when pooled and assayed for enzyme activity showed that approximately 80-90% of the initial arginase activity was present.

(b) *Sephadex G-200 treatment.*

Gel filtration of cell-free extracts was carried out



using Sephadex G-200 by the method described by Andrews (1965). Samples of highly purified proteins, of known molecular weights (Mann Research Laboratories) were used to obtain standard elution volumes. The positions of these proteins in the effluent from the column were determined on the basis of extinction measurements at appropriate wavelengths (Andrews, 1965).

Before use, the columns were equilibrated with 0.01 M potassium phosphate buffer (pH 7.5). 0.5 ml of the cell-free extract, containing approximately 10 mg of protein, was applied to the top of the column by layering under a small volume of buffer already present. Pressure on the column was maintained by using a LKB Perpex peristaltic pump (Model 10200) connected to the top of the column. In this way a steady flow rate of 20 ml/hr was obtained during elution. All experiments were carried out at 4°C. Before samples were applied to the column, uniformity of column packing was checked by passage of a sample of Blue Dextran 2000 through the column. The column effluent was collected in fractions of 3 ml. Each tube was assayed, quantitatively, for protein content (see later section) as well as scanned at 280 mn using a Beckman DB-G spectrophotometer.

#### *Assay of arginase activity*

Except as mentioned below, arginase activity in either the initial cell-free extract or in the partially purified enzyme preparations was assayed using the following reaction



system. Enzyme activation was achieved by pre-incubation with 10  $\mu$ moles manganese maleate (pH 7.0) for 30 minutes at 30°C. Arginase activity was then assayed by incubation of activated enzyme with 140  $\mu$ moles of L-arginine (free base) pH 9.2. The final volume of the reaction system was 0.5 ml. After incubation for 10 minutes at 30°C, the reaction was stopped by adding 4 ml of Hycel urea nitrogen reagent. The color was developed for 12 minutes in a boiling water bath followed by immediate chilling of the tubes for 3 minutes in an ice bath. The yellow color developed with urea was measured at 490 nm. Control systems, which did not include either enzyme or substrate, were included. The final color yield was corrected for these controls. The quantity of urea liberated was determined by reference to a standard curve prepared under the test conditions with a standard urea solution.

In kinetic and inhibitor studies, arginase activities were assayed by a slight modification of Middelhoven's method (Middelhoven, 1969). In these cases, arginase was assayed in the following manner. Equal amounts of 40 mM manganese maleate buffer (pH 7.0) and 40 mM TrisHCl (pH 7.0) were mixed to form  $Mn^{++}$ -TrisHCl buffer (pH 7.0). The partially purified enzyme (0.1 ml), approximately 0.1 mg protein, was activated in 0.1 ml of  $Mn^{++}$ -TrisHCl solution at 50°C for 15 minutes. 4  $\mu$ moles of sodium glycinate buffer (pH 7.0) and 100  $\mu$ moles of L-arginine (pH 9.2) were then added to make a total volume of 0.5 ml. The whole mixture was then



incubated at 30°C for 10 minutes. The reaction was stopped by addition of Hycel urea reagent as described above.

One unit of arginase activity is defined as the amount of enzyme producing 1  $\mu$ mole of urea or ornithine per hour at 30°C under the reaction conditions specified. Specific enzyme activity is expressed as units of enzyme activity per mg of protein.

#### *Assay of ornithine transcarbamylase activity*

The following reaction system was used in assaying ornithine transcarbamylase activities. The partially purified enzyme was incubated with 200  $\mu$ moles of TrisHCl buffer (pH 8.5), 50  $\mu$ moles of L-ornithine·HCl (pH 8.5) and 20  $\mu$ moles each of carbamyl phosphate and  $MgCl_2$ . The total volume was 3 ml and incubation was for 30 minutes at 30°C. The citrulline produced in the reaction was assayed by the method of Grisolia and Ratner (1955) as modified by Shafer and Thompson (1967). The reaction was stopped by adding 4 ml of an acid mixture, made by mixing 0.237 g  $MnSO_4 \cdot H_2O$  in 398 ml of distilled water with 1.8 ml 0.1 M  $FeCl_3$  solution, 300 ml concentrate  $H_3PO_4$  and 100 ml concentrate  $H_2SO_4$ , technical grade. Without delay, 0.25 ml of 3% aqueous diacetylmonoxime solution was added followed by thorough mixing. The tubes were capped and heated in boiling water for 15 minutes in darkness. The tubes were cooled in tap water in darkness for 10 minutes and absorbance at 490 nm was determined within 20 minutes. Corrections were made from



reagent blanks which were mainly due to traces of urea in the carbamylphosphate. One unit of enzyme activity is defined as the amount of enzyme producing 1  $\mu$ mole of citrulline in 1 hour at 30°C under the reaction conditions specified. Specific enzyme activity is expressed as units of enzyme activity per mg protein.

#### *Assays of argininosuccinate lyase activity*

The assay was according to Ratner *et al.* (1953). The reaction system consisted of, in a total volume of 0.5 ml, partially purified enzyme preparation (after Sephadex G-25 treatment), 2  $\mu$ moles barium L-argininosuccinate, 10  $\mu$ moles  $K_2SO_4$ , 50  $\mu$ moles potassium phosphate (pH 7.4) and 1 mg of bovine liver arginase (Sigma). The mixture was incubated for 30 minutes at 30°C. The reaction was stopped with 4 ml of Hycel urea nitrogen reagent. The primary reaction products were fumarate and arginine. The latter, after conversion to urea by the addition of arginase, was assayed as described earlier. One unit of enzyme activity is defined as the amount of enzyme catalyzing the production of 1  $\mu$ mole of arginine per hour under the defined experimental conditions. Specific enzyme activity is expressed as units of enzyme activity per mg protein.

#### *Assay of ornithine $\delta$ -transaminase*

The spectrophotometric assay of this enzyme depends on the hydrolysis of L-ornithine, formed in the enzymatic



reaction, to glutamic  $\gamma$ -semialdehyde, which cyclizes to form  $\Delta'$ -pyrroline-5-carboxylic acid and then reacts with  $\alpha$ -amino-benzaldehyde to yield a yellow dihydroquinazolinium compound (Albrecht and Vogel, 1964; Middelhoven, 1964). The standard enzymatic reaction mixture contained, in a total volume of 0.5 ml, 50  $\mu$ moles of potassium phosphate buffer (pH 7.5), 2  $\mu$ moles L-ornithine, 2  $\mu$ moles of  $\alpha$ -ketoglutarate, 0.01  $\mu$ mole of pyridoxal-5'-phosphate, and enzyme preparation containing approximately 100-200 mg protein. After an incubation period of 30 minutes at 30°C, the reaction was stopped by addition of 0.3 ml 6N HCl. Two types of controls were included, firstly, a reference blank from which the enzyme was omitted and secondly, a control from which L-ornithine was initially omitted but supplied following the addition of hydrochloric acid. The tubes were then covered with aluminium foil and heated in boiling water bath in darkness for 30 minutes. After being cooled to 25°C, 1 ml of 3.6 M aqueous sodium acetate and 0.2 ml of 0.033 M aqueous  $\alpha$ -aminobenzaldehyde were added to the mixture. The tubes were allowed to stand for 15 minutes at 25°C. The absorbance of the resulting yellow solution was determined in a Beckman spectrophotometer, in quartz cuvettes with a 1 cm light path, at 440 nm. The solution was clarified by centrifugation prior to absorbance measurement, if necessary.

One unit of  $\delta$ -ornithine transaminase is defined as that amount of the enzyme which will yield an absorbance of 0.100 when tested under the standard conditions of enzyme assay.



This absorbance value corresponds to 0.086  $\mu$ moles of synthetic  $\Delta'$ -pyrroline-5-carboxylic acid hydrolyzed per reaction mixture (Strecker, 1960). Specific enzyme activity is expressed as units of enzyme activity per mg protein. Data presented in the Results section for all enzyme assays are average values derived from measurements made in at least triplicate. Variations within different experiments were consistently within  $\pm 10\%$ .

#### *Estimation of protein content of enzyme preparations*

The protein content of enzyme preparations was estimated colorimetrically as described by Lowry *et al.* (1951).

Crystalline egg albumum was used as a reference standard.

#### *Radioisotopic assay of arginase activity*

L-arginine (guanido- $^{14}\text{C}$ ) was used in a radioisotopic assay of arginase activity in yeast extracts. This enabled a study of the effect of urea and L-ornithine on the rate of arginine cleavage in the enzymatic reaction. The method was a modification of that described by Righetti *et al.* (1967).

This is based on the formation of  $^{14}\text{C}$ -urea from guanido-labelled arginine, followed by chemical cleavage of this reaction product with sodium nitrite and HCl to give  $^{14}\text{CO}_2$ . The latter is collected and assayed for  $^{14}\text{C}$ .

Incubations of arginase preparations and substrate were performed as described previously in this section. The reaction systems were incubated in 25 ml Warburg flasks fitted with a side arm and having a centre well. The substrate was prepared by mixing 4 ml of 0.7 M L-arginine solution (pH 9.5) with 50  $\mu$ l of L-arginine (guanido- $^{14}\text{C}$ ) which had a specific activity of 4.58  $\mu\text{c}/\mu\text{mole}$ . In every



assay, approximately 0.3 - 0.5 mg of protein (Sephadex G-25 treated enzyme) and 280  $\mu$ moles of L-arginine were incubated together. After incubation, the reaction was terminated by adding 1 ml of 1N HCl. Then 1 ml of 5% NaNO<sub>2</sub> solution was placed in the side arm, and 0.3 ml of Hyamine hydroxide (50% aqueous solution) was placed in the centre well. The flasks were sealed with glass stoppers and the NaNO<sub>2</sub> solution in the side arm was tipped into the reaction mixture. During liberation of  $^{14}\text{CO}_2$ , the flasks were placed in a Dubnoff metabolic shaking incubator (Precision Scientific Company, Chicago, U.S.A.) operated at 38°C. Complete absorption of  $^{14}\text{CO}_2$  was ensured by shaking the flasks for one hour. During the last 10 minutes the temperature was raised to 50°C.

#### *Measurement of absorbed $^{14}\text{CO}_2$*

When CO<sub>2</sub> liberation was completed, the flasks were opened and the hyamine solution withdrawn to a liquid scintillation vial containing 15 ml of the following solution: 400 ml of 0.6% PPO in toluene and 300 ml of absolute ethanol (Righetti *et al.*, 1968). The vials were shaken vigorously for a few seconds to mix the contents, and the centre wells in the Warburg flasks were rinsed several times with small aliquots of the scintillation fluid. The vials were then counted under predetermined balance point conditions in a liquid scintillation counter (Nuclear Chicago Corp., Unilux II Model). Control reaction systems, containing enzyme, were routinely included in all such



assays. The counting efficiency was approximately 76% as determined by the channels ratio method. All counts were corrected for background.

#### *Cycloheximide feedings experiments*

Cells harvested in the exponential growth phase from 2 litres of basic culture medium containing amino acids were used for inoculation of two experimental cultures. One contained the basic culture medium supplemented with  $2 \times 10^{-2}$  M L-arginine while the other contained the basic culture medium supplemented with  $2 \times 10^{-2}$  M urea. Cells were then harvested from each at the end of a five hour incubation period at 30°C, and washed three times with cold distilled water. One quarter of these cells were sonicated as described earlier, subjected to the Sephadex G-25 treatment and assayed for arginase activity. The rest of the cells were resuspended in 500 ml of the basic culture medium supplemented with  $2 \times 10^{-2}$  M urea. Cycloheximide, at a concentration of 10  $\mu$ g/ml, was added as indicated in the Results Section. Aliquots of the cultures (100 ml) were collected after different periods during growth, chilled in an ice bath, centrifuged, and washed three times with ice-cold distilled water. These cells were then disintegrated by sonication treatment, the extracts passed through Sephadex G-25 and assayed for arginase activities. In all cases, growth of the cultures was measured spectrophotometrically using a Beckman spectrophotometer as mentioned previously.



*Free amino acid determinations*

Levels of citrulline, arginine and urea, in the culture media and in cell extracts, were determined by colorimetric assays as described in detail in an earlier section of this thesis (page 33). Levels of free amino acids, ammonia and urea, both in the culture media after incubation and in extracts of cells grown in various media, were determined in a Beckman Automatic Amino Acid Analyzer, Model 121. For such analyses, the free amino acids in cell extracts were isolated by the following method. Cells from 100 ml aliquots of the appropriate culture medium were harvested by centrifugation, washed and resuspended in 2 ml of cold distilled water. The suspensions were then sonicated and heated in a boiling water bath for 6 minutes. The temperature of the cell suspension was rapidly raised to about 85°-90°C during this time period. Denatured protein and cell debris were removed by centrifugation at 18,000 x g for 10 minutes. The resulting supernatants were passed through columns (1x6 cm) of analytical grade cation exchange resin, Dowex 50W-X8 ( $H^+$  form) 100-200 mesh (Bio Rad Laboratories, Richmond, California, U.S.A.). The columns were washed with 40 ml of deionized water and the column effluents were discarded. The amino acids were eluted by addition of 30 ml of 2N HCl. The eluted HCl, containing the amino acids, was then dried *in vacuo* in a Flash-Evaporator (Buchler Instrument, Fort Lee, N.J., U.S.A.). The dried residue was finally dissolved in 2 ml of 0.2 M citrate buffer (pH 2.2). This



amino acid extract was then subjected to analysis in the Amino Acid analyzer using UR-30 and PA-35 spherical resins (Beckman Instruments, Inc., California, U.S.A.). The eluting buffers for separating the neutral and acidic amino acids were 0.20 M citrate buffer (pH 3.22 and pH 4.25). The basic amino acids were eluted from the PA-35 resin using 0.35 M citrate (pH 5.25). The concentration of each amino acid present was determined by the HxW method. The pH values of all buffers were measured at 22°C.



## RESULTS

*Growth of yeast cells in various culture media*

Before any attempts were made to study regulation of Kreb-Henseleit cycle enzymes, it was necessary to obtain information regarding the growth of *Saccharomyces cerevisiae* under different culture conditions. Growth was monitored by taking aliquots of the cells from the media at various time intervals, making appropriate dilutions, and measuring optical densities as described in the Materials and Methods. The results of these experiments (Figure 3) show that cells grown in the basic culture medium supplemented with the amino acid solution (10 ml/litre culture medium) were growing exponentially 12 hours after inoculation and furthermore this phase of growth was maintained for more than 30 hours. Such cells were harvested during the exponential phase (24 hours from inoculation) and used to inoculate a basic culture medium supplemented with  $2 \times 10^{-2}$  M L-arginine and  $2 \times 10^{-2}$  M urea respectively (Figures 4a and 4b). Considering the change in optical density with time, it is possible to identify a lag phase, between time 0 and 2 hours in both cultures. A phase of logarithmic growth, occurred between the 2nd and the 15th hour in case of the L-arginine supplemented culture (Figure 4a) and between the 2nd and 18th hour in case of urea supplemented culture (Figure 4b). Post-exponential phases were obvious after approximately 20 hours in both cultures (Figures 4a and 4b). When the





FIGURE 3

*GROWTH CURVE OF YEAST CELLS IN BASIC CULTURE MEDIUM*

*SUPPLEMENTED WITH AMINO ACID SOLUTION*

(10 ml per litre)

All readings were made after a 20-fold dilution. The data represent average values from four separate experiments.

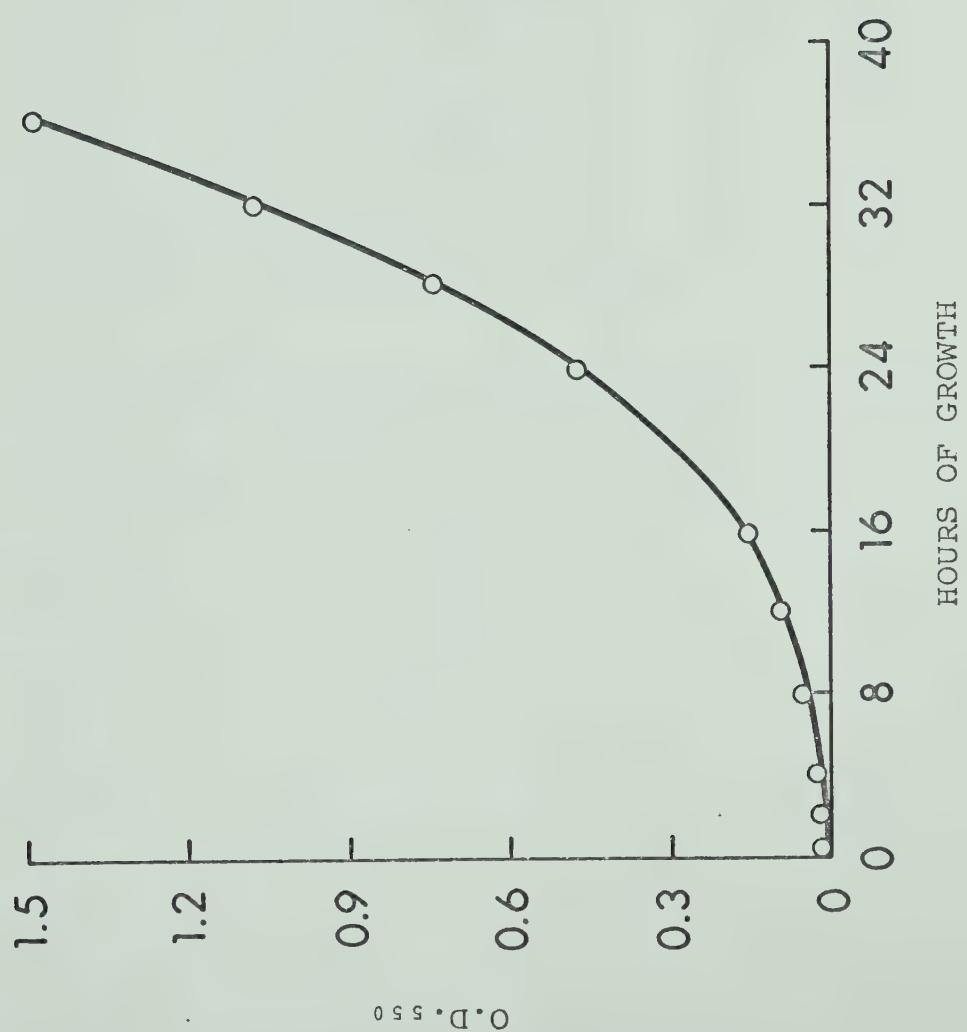






FIGURE 4a

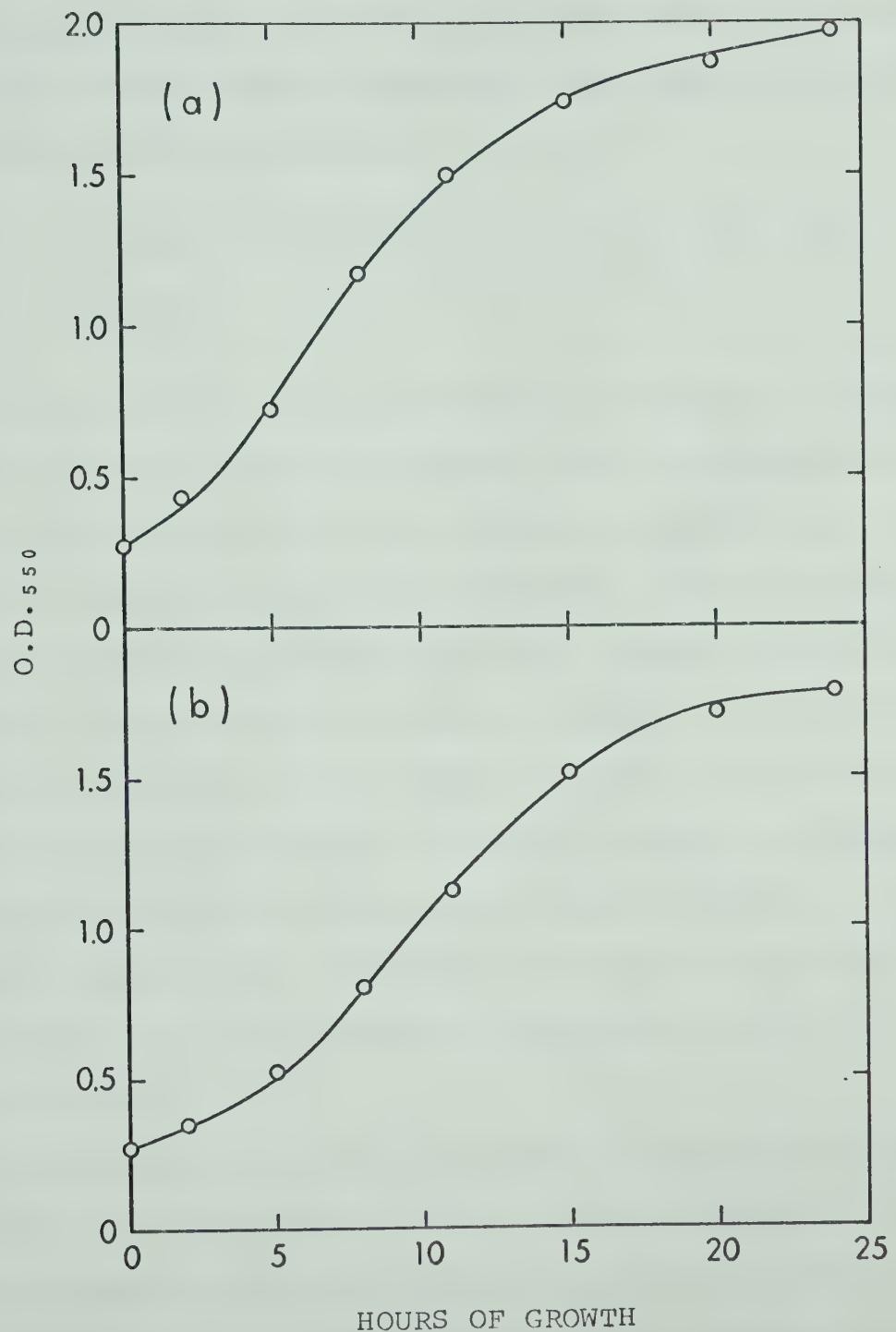
GROWTH CURVE OF YEAST CELLS IN BASIC MEDIUM SUPPLEMENTED WITH  
 $2 \times 10^{-2} M$  UREA

FIGURE 4b

GROWTH CURVE OF YEAST CELLS IN BASIC MEDIUM SUPPLEMENTED WITH  
 $2 \times 10^{-2} M$  L-ARGININE

In each case the cells were initially grown with aeration in the basic medium supplemented with the amino acid solution (10 ml per litre). After 24 hours at 30°C, samples of the cells were transferred immediately to the above media and harvested at intervals as indicated. All readings were made after a 10-fold dilution.

The data represent average values from three separate experiments.





slopes of the exponential phases in Figures 4a and 4b are compared, it is obvious that the cells cultured in the presence of exogenous urea grew slightly more rapidly than those in the arginine medium. In both cases, high optical densities were reached indicating that these nitrogen sources were utilized by the cells.

*Effects of some nitrogen-rich compounds on the levels of arginase in vivo*

As outlined in the introduction, enzymes of the Krebs-Henseleit cycle in microorganisms, are scrupulously controlled. It is well known that arginine represses most of the enzymes catalyzing arginine synthesis. This is especially well documented for *Escherichia coli* (Vogel, 1961; Forsyth *et al.*, 1969). In *Saccharomyces*, Bechet *et al.* (1962) and Middelhoven (1969) showed that ornithine transcarbamylase was repressed by arginine. However, possible effects on arginase activities were not examined in detail. In the present studies, the influence of some nitrogen-rich compounds, on the levels of arginase in yeast cultures, was examined.

In order to determine changes in the levels of arginase present in crude extracts, the cells were harvested at the end of growth periods on various supplemented media as shown in Table III. It is clear that specific enzyme activities differed in cells grown in the presence of different nitrogen compounds. L-arginine, at a concentration of



$2 \times 10^{-2} M$ , gave the highest specific enzyme activity while urea and  $(\text{NH}_4)_2\text{SO}_4$ , both present at  $2 \times 10^{-2} M$ , gave the lowest specific enzyme activities. Cells cultures without a nitrogen supplement were found to have a specific enzyme activity between these extremes. When the arginine concentration was increased from  $2 \times 10^{-2} M$  to  $2 \times 10^{-1} M$ , the specific activity of arginase decreased from 31.80 to 9.60. This may be due to an accumulation of urea formed from arginine as addition of urea to the basic medium resulted in decreases in the specific activity of arginase. Various amounts of arginine added with ornithine, urea or  $\text{NH}_2\text{SO}_4$ , tended to increase the levels of arginase to some extent (Table III). Ornithine alone, when present in the culture media, decreased the enzyme activity below that of the control. Thus, the products of arginase activity appeared to exert a repressing or inhibiting effect on the activity of this enzyme while the substrate, L-arginine, appeared to induce increases in enzyme activity when added in low concentration.

#### *Derepression of arginase by arginine supplements*

Following the preliminary studies described above, it was of interest to determine what changes in arginase levels would occur when cells, grown initially on media containing  $2 \times 10^{-2} M$  L-arginine and  $2 \times 10^{-2} M$  urea, were inoculated into media supplemented with arginine in different concentrations and with a combination of L-arginine and L-citrulline or



TABLE III

THE INFLUENCE OF ARGinine, ORNITHINE, UREA AND  $(NH_4)_2SO_4$  ON THE  
SPECIFIC ACTIVITY OF ARGINASE

Basic culture medium supplement	Concentration	Specific activity ( $\mu$ moles urea/hr/mg protein)
None		11.30
L-arginine	$2 \times 10^{-2}M$	31.80
L-arginine	$2 \times 10^{-1}M$	9.60
Urea	$2 \times 10^{-2}M$	4.40
Urea	$2 \times 10^{-1}M$	3.00
L-ornithine	$2 \times 10^{-2}M$	8.00
$(NH_4)_2SO_4$	$2 \times 10^{-2}M$	4.20
L-arginine-HCl + urea	$2 \times 10^{-2}M$	5.10
L-arginine-HCl + L-ornithine	$2 \times 10^{-2}M$	23.4
L-arginine-HCl + $(NH_4)_2SO_4$	$2 \times 10^{-2}M$	7.20

Cells were grown for 4 hours in the media indicated. Data were derived from three separate experiments.



L-ornithine. Table IV shows that as before cultures grown in the presence of  $2 \times 10^{-2}$  M L-arginine contained the greatest enzyme activity. However, in this experiment it is clear that within 5 hours of transfer from a urea containing medium, the levels of arginase had risen substantially. This observation is highly suggestive of a derepression of arginase under these new culture conditions. These changes were readily reversible on transfer of such cells to suitable media containing urea as described in a later experiment.

These data, therefore, confirm the studies made by Middelhoven (1964, 1968) who demonstrated that arginine readily induced arginase and that repression of this enzyme occurred in the presence of ammonium salts. It is also clear from this experiment (Table IV) that yeast cells when grown in an environment rich in carbon sources, but devoided of exogenous nitrogen, are capable of maintaining a certain level of arginase activity above that of urea grown cells. This level of enzyme activity was elevated or depressed depending on the presence of certain nitrogen compounds in the culture environment.

In a further experiment, the derepression of arginase by arginine was followed as a function of time (Table V). Within 30 minutes of transfer from a urea containing medium the specific activity of arginase rose more than five times. This was followed by further increases until at 6 hours the specific activity of arginase was fourteen times that of the urea grown cells.



TABLE IV

*DEREPRESSION OF ARGINASE FOLLOWING GROWTH  
IN THE ABSENCE OF UREA*

Supplement to basic culture medium	Specific activity ( $\mu$ moles urea/hr/mg protein)
None	11.0
L-arginine ( $1 \times 10^{-1}$ M)	9.60
L-arginine ( $2 \times 10^{-2}$ M)	16.60
L-arginine + L-citrulline ( $2 \times 10^{-2}$ M)	12.40
L-arginine + L-ornithine ( $2 \times 10^{-2}$ M)	12.80

Cells were initially cultured in the basic medium supplemented with  $2 \times 10^{-2}$ M urea and  $2 \times 10^{-2}$ M L-arginine for 24 hours (specific enzyme activity 1.2), followed by transfer to the indicated media. Cell-free extracts were prepared after 5 hours of growth. Data were derived from three separate experiments.



TABLE V

*DEREPRESSION OF ARGINASE BY 2 X 10<sup>-2</sup>M L-ARGININE  
AS A FUNCTION OF GROWTH TIME*

Culture period (hours)	Specific activity of arginase ( $\mu$ moles urea/hr/mg protein)
0	1.2
0.5	6.6
1	8.0
1.5	8.5
2	9.6
2.5	10.6
3	10.8
3.5	11.0
4	13.2
5	15.0
6	16.8

The yeast cells were initially cultured in the basic culture medium supplemented with  $2 \times 10^{-2}$ M urea for 4 hours before transfer to the arginine supplemented medium. The data represent the mean of 2 separate experiments.



It appeared likely that these changes of arginase activity were reversible on transfer of the cells to suitable media (Tables VII and VIII) and time-course experiments (Table V) suggested that repression and derepression of this enzyme may be involved. When cells are grown on a basic culture medium, arginase is synthesized at a constant "normal" rate. On addition of urea further formation of the enzyme immediately ceases, and the content of the enzyme per cell gradually decreases as it is diluted by growth. This was shown by the decrease of specific activity of the enzyme determined on basis of protein concentration. When the urea was exhausted or arginine added, the enzyme was synthesized again (Table VIII). However, instead of taking several generations to build up the normal level, there is an explosive preferential synthesis of the enzyme which restores the "normal" level in a very short time (Table V).

#### *Column chromatography with Sephadex G-25*

When cell-free extracts of yeast cells were passed through columns of Sephadex G-25, the specific activities of arginase were increased by a factor of 6.4 (Table VI). This increase occurred irrespective of the culture conditions employed for growth of the cells. These observations, therefore, support the contention that changes in the specific activity of arginase are the result of the changes in the rate of synthesis *de novo*. If, alternatively, the decrease in arginase when urea or ammonium salts are present in the



TABLE VI

## SPECIFIC ACTIVITIES OF ARGINASE PREPARATIONS AFTER DESALTING WITH SEPHADEX G-25

Basic culture medium supplemented with:	Specific activity of arginase ( $\mu$ moles urea/hr/mg protein)		Ratio of specific activities (initial/final)
	Before Sephadex G-25	After Sephadex G-25	
L-arginine ( $2 \times 10^{-2}$ M)	28.0	178.0	6.4
Urea ( $2 \times 10^{-2}$ M)	4.30	27.4	6.4
$(\text{NH}_4)_2\text{SO}_4$ ( $2 \times 10^{-2}$ M)	4.20	26.8	6.4

Extracts were prepared from cells grown for 4 hours in the above media.

Arginase was assayed under the standard reaction conditions. The enzyme was assayed in duplicate, and the data represent the average of 3 separate experiments.



TABLE VII

REPRESSION OF ARGINASE AS A FUNCTION OF UREA CONCENTRATION IN THE CULTURE MEDIUM \*

Concentration of urea added to the basic culture medium** (μmoles urea/hr/mg protein)	Specific activity of arginase	Growth of cells g.d.wt/litre of culture	Specific activity x g.d.wt/litre
$2 \times 10^{-4} M$	86.0	0.58	50.0
$2 \times 10^{-3} M$	42.0	0.60	25.0
$2 \times 10^{-2} M$	14.0	0.72	10.0
$6 \times 10^{-2} M$	13.0	0.62	8.0
$1 \times 10^{-1} M$	12.0	0.62	7.5
$2 \times 10^{-1} M$	10.0	0.60	6.0

\* Arginase levels were assayed after Sephadex G-25 treatment.

\*\* Cells were grown in basic culture medium supplemented with  $2 \times 10^{-2} M$  L-arginine for 4 hours (specific enzyme activity 140.0) before transfer to the above media. Extracts were prepared after 4 hours growth in the new supplemented media. The enzyme was assayed in duplication, and the data represent the average of 3 separate experiments.



TABLE VIII

REPRESSION OF ARGINASE AS A FUNCTION OF ARGinine CONCENTRATION IN THE CULTURE MEDIUM\*

Concentration of L-arginine added to the basic culture medium**	Specific activity of arginase (μmoles urea/hr/mg protein)	Growth of cells g.d.wt/litre of culture	Specific enzyme activity x g.d.wt/litre
$2 \times 10^{-4} M$	64.0	0.43	27.50
$2 \times 10^{-3} M$	89.0	0.43	38.0
$2 \times 10^{-2} M$	111.0	0.42	47.0
$6 \times 10^{-2} M$	72.0	0.39	28.0
$1 \times 10^{-1} M$	34.0	0.36	12.0
$2 \times 10^{-1} M$	20.0	0.33	6.0

\* Arginase levels were assayed after Sephadex G-25 treatment.

\*\* Cells were grown in basic culture medium supplemented with  $2 \times 10^{-2} M$  urea for 4 hours (specific enzyme activity 11.5) before transfer to the above media. Extracts were prepared after 4 hours growth in the new supplemented media. The enzyme was assayed in duplicate, and the data represent the average of 3 separate experiments.



culture was due to an inhibitory effect of these molecules on the action of the enzyme then such an inhibition would theoretically be removed by gel treatment. However, gel treatment did not raise the specific activity of urea grown cells to that of arginine grown cells even though such treatment was found, in independent experiments, to remove such low molecular weight compounds from protein.

*Changes in the levels of arginase in response to arginine and urea concentrations*

In order to examine, in more detail, the effects of urea and arginine on the levels of arginase, various concentrations of these compounds were used to supplement the culture media. Cells initially supplied with  $2 \times 10^{-2}M$  arginine were found to contain the highest arginase (Table VII). When transferred to media containing various levels of urea, followed by a 4 hour growth period, dramatic decreases in the specific activity of arginase occurred. For cultures containing  $2 \times 10^{-2}M$  urea had specific activities which were one-tenth of the initial values. Above this concentration of urea, smaller but less significant decreases were observed. In another experiment, the cells were initially cultured in the presence of  $2 \times 10^{-2}M$  urea. After 4 hours of growth these cultures had low levels of arginase (Table VIII). Following a further 4 hour growth period in the presence of various concentrations of arginine, the levels of arginase were increased. Again approximately



10-fold changes were observed depending on the concentration of arginine supplied. As in the earlier experiments (Tables III and IV), arginase levels were decreased when concentrations of arginine, above  $2 \times 10^{-2} M$ , were present in the culture medium.

Although the data presented so far indicates that arginine and urea are altering the rate of synthesis of arginase, the possibility remains that the observed changes in specific activity may be in large part due to changes in total protein concentration rather than the actual synthesis of the enzyme. In other words, the enzyme level may remain relatively constant but changes in total soluble protein may tend to alter the specific enzyme activity due to dilution or concentration effects. To test this possibility, total dry weight of the cells was determined and this value was multiplied by the specific enzyme activities in each case (Tables VII and VIII). From these Tables it is obvious that the values obtained differ greatly. Clearly, if urea and arginine altered the production of protein without changing the levels of arginase activity, these values should be very similar (Middelhoven, 1969).

*The effects of urea and ornithine on arginase activity in vivo and in vitro*

As described earlier, the two end-products of arginine breakdown, namely urea and ornithine, were found to have a profound influence on the activity of arginase. Middelhoven



(1964) in his work with yeast arginase, discovered that when 10 mM L-ornithine·HCl were added to the culture medium, the specific activity was only 50% of that found in cultures supplemented with the same concentration of L-arginine·HCl. When 20 mM ammonium sulfate was added to culture media containing 10 mM L-ornithine·HCl, the specific activities of arginase were further reduced from 68 to 18. This compared to a specific activity of 125 when the cells were cultured in a medium containing 10 mM L-arginine·HCl. Similar results were obtained in the present experiments as summarized in Tables III and IV. However, it is interesting to note that when such enzyme preparations were treated with Sephadex G-25, the effect caused by L-ornithine was removed and the specific activity was increased (Table IX). When enzyme preparations from media supplemented with different sources of nitrogen were treated by gel filtration, the specific activities were increased by a factor of 5-7. However, when the initial extract of cells grown on an arginine-rich medium was assayed in the presence of 300  $\mu$ moles of L-ornithine, the specific enzyme activity was decreased to 4, a value similar to that obtained for cells grown in the presence of urea. However, when such ornithine pre-treated extracts were passed through columns of Sephadex G-25, the specific activity increased by a factor of 37. This shows that the inhibitory effect of ornithine was removed by Sephadex G-25 treatment. This strongly indicates that under these *in vitro* conditions ornithine exerts its



TABLE IX

## CHANGES IN SPECIFIC ACTIVITIES OF ARGINASE PREPARATIONS AFTER GEL FILTRATION

Supplement to the basic culture medium	Concentration Conditions	Specific activity of arginase (μmoles urea/hr/mg protein)		
		Assay	Before Sephadex G-25 treatment	After Sephadex G-25 treatment
None	standard	10.6		65
L-arginine	$2 \times 10^{-2}$ M	"	29	164
Urea	$2 \times 10^{-2}$ M	"	3	20
L-ornithine-HCl	$2 \times 10^{-2}$ M	"	7	31
L-arginine	$2 \times 10^{-2}$ M	"	19	121
+ L-ornithine-HCl	$2 \times 10^{-2}$ M	plus 300 μmoles L-ornithine*	4	148
L-arginine	$2 \times 10^{-2}$ M			

\*In this experiment, 2 samples of cell-free extract were preincubated with 300 μmoles L-ornithine each at 30°C for 10 minutes. Of these 2 samples, one was assayed for arginase with L-arginine as substrate after the preincubation period, while the other was subjected to Sephadex G-25 treatment before assay for arginase. All assays were carried out in duplicate and the values obtained were averages of at least 3 separate experiments.



effect by inhibiting enzyme activity rather than repressing enzyme synthesis. Some evidence for the latter is, however, given by the data for the ornithine supplemented cultures (Table IX). As before (Table III), the specific activity under these conditions was slightly lower than the control; but this level was increased to the same extent as the other preparations by gel filtration.

#### *Radioisotopic studies of arginase activities*

As the above data indicates that ornithine and urea affect the arginase activity differently *in vitro* and *in vivo*, it was of interest to compare how these two substances affected this enzyme. As addition of urea to the reaction system would prevent accurate measurement of arginase by the colormetric procedure, a sensitive radioisotopic assay was employed in these experiments.

L-arginine (guanido<sup>14</sup>C) was used as a substrate in these experiments and was added to three different reaction systems. The first one contained a partially purified enzyme preparation extracted from yeast cells grown in the presence of  $2 \times 10^{-2}$ M arginine. The substrate was 280  $\mu$ moles L-arginine solution containing 0.5  $\mu$ c of <sup>14</sup>C. The second reaction system was the same as the first except 100  $\mu$ moles of urea were added in addition, and the third contained 100  $\mu$ moles of L-ornithine. The results presented in Table X represent separated experiments. It is clear that urea had no effect on arginase activity while L-ornithine



TABLE X

THE EFFECT OF UREA AND ORNITHINE ON THE ACTIVITY OF  
YEAST ARGINASE

Reaction System	Urea formed ( $\mu$ moles)
Enzyme + arginine- $^{14}\text{C}$	8.2
Enzyme + arginine- $^{14}\text{C}$ + 100 $\mu$ moles urea	7.7
Enzyme + arginine- $^{14}\text{C}$ + 100 $\mu$ moles L-ornithine	2.2

Arginase activity was measured using arginine (guanido- $^{14}\text{C}$ ) as described in the Materials and Methods. Cell-free extracts of yeast cells grown in the presence of  $2 \times 10^{-2}\text{M}$  arginine were used as a source of the enzyme. All assays were carried out in triplicate and the values obtained were averages of at least 3 separate experiments.



inhibited the enzyme to a great extent. In a further experiment (Table XI) it was confirmed that urea did not appreciably affect arginase activity even when added to a level of 100  $\mu$ moles. In contrast similar increases in the levels of ornithine added to the reaction system gave progressive inhibition of enzyme activity.

*The effect of cycloheximide on the repression of arginase by urea*

Cycloheximide is an antibiotic originally isolated from cultures of *Streptomyces griseus* by Whiffen *et al.* in 1946. For the present experiment, this compound was purchased from Sigma Chemical Company. The effect of cycloheximide on the metabolism of yeast is to cause a cessation of protein synthesis and an inhibition of DNA synthesis (Siegel and Sisler, 1963). However, the synthesis of many organic acids and amino acids is not inhibited in the presence of the antibiotic.

In the present experiment, cycloheximide, at a concentration of 10  $\mu$ g/ml was added to samples of the culture media which had been supplemented with urea. Initially the cells were harvested from 5 hour incubations in either an arginine-rich medium or a urea-rich medium. Such cells were then used to inoculate in fresh urea-rich culture media containing 10  $\mu$ g/ml antibiotic. Growth was measured spectrophotometrically. It is clear from Figure 5 that cycloheximide effectively inhibited growth of the cells. When the



TABLE XI

THE EFFECT OF DIFFERENT CONCENTRATIONS OF UREA AND ORNITHINE  
ON THE ACTIVITY OF YEAST ARGINASE

Standard reaction system + urea (μmoles)	Urea formed (μmoles)	Standard reaction system + ornithine (μmoles)	Urea formed (μmoles)
0	8.0	0	8.4
20	7.7	20	7.2
40	7.8	40	6.0
60	8.1	60	4.8
80	7.4	80	3.5
100	7.6	100	1.9

The reaction systems contained the same enzyme preparations and substrate levels as indicated in Table X. Various concentrations of urea and ornithine were added as indicated. The data are average values obtained from determinations made in triplicate.



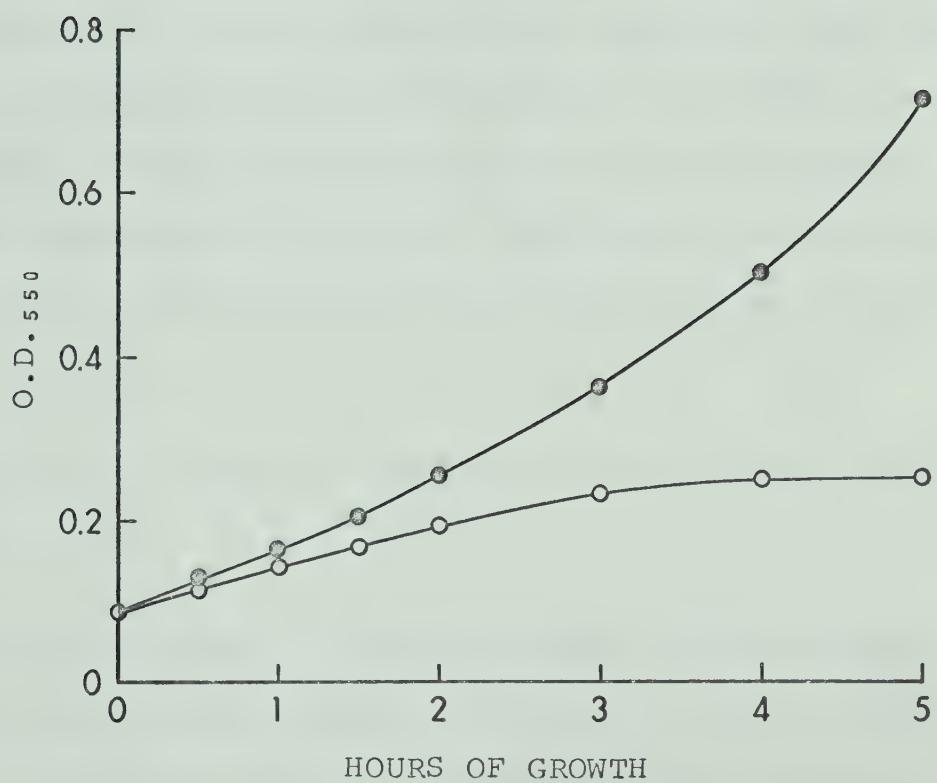


## FIGURE 5

*THE EFFECT OF CYCLOHEXIMIDE ON THE GROWTH OF YEAST CELLS*

The cells were initially grown in basic culture medium supplemented with  $2 \times 10^{-2}$ M urea for 5 hours at 30°C before being inoculated into a fresh basic culture media supplemented with  $2 \times 10^{-2}$ M urea, in the presence (o - o) and absence (o - o) of cycloheximide. All readings were taken after a 10-fold dilution.

The data are average values from three separate experiments.





accompanying changes in arginase were examined (Figures 6 and 7), the following major observations were made. As in earlier experiments, cells cultured in the presence of urea possessed low levels of arginase which decreased further during culture under these conditions. However, these decreases were not observed when cycloheximide was present in the medium. Under these conditions, the specific activity of arginase rose, the rise being related to the addition of this antibiotic. However, when these increases were compared with the increases caused by L-arginine (*c.f.* Tables IV and VIII), where the specific activity of arginase increased approximately 10-fold, the present increases in enzyme activity associated with cycloheximide are slower and smaller.

*Gel filtration of arginase extracted from arginine- and urea-grown cells*

The data obtained in the proceeding cycloheximide experiment are in many respects similar to observations made by Bechet and Wiame (1965) on changes in OTC activity. These authors concluded that synthesis of protein was essential for regulation of OTC in yeast and in a later publication (Messenguy and Wiame, 1969), reported the chromatography of a regulatory protein which combined with OTC and, therefore, controlled its catalytic activity. The complex so formed differed in molecular weight from 'free' OTCase and was, therefore, distinguishable by gel filtration. As the





## FIGURE 6

*THE EFFECT OF CYCLOHEXIMIDE ON THE ACTIVITY OF ARGINASE OF CELLS INITIALLY CULTURED IN THE PRESENCE OF L-ARGININE*

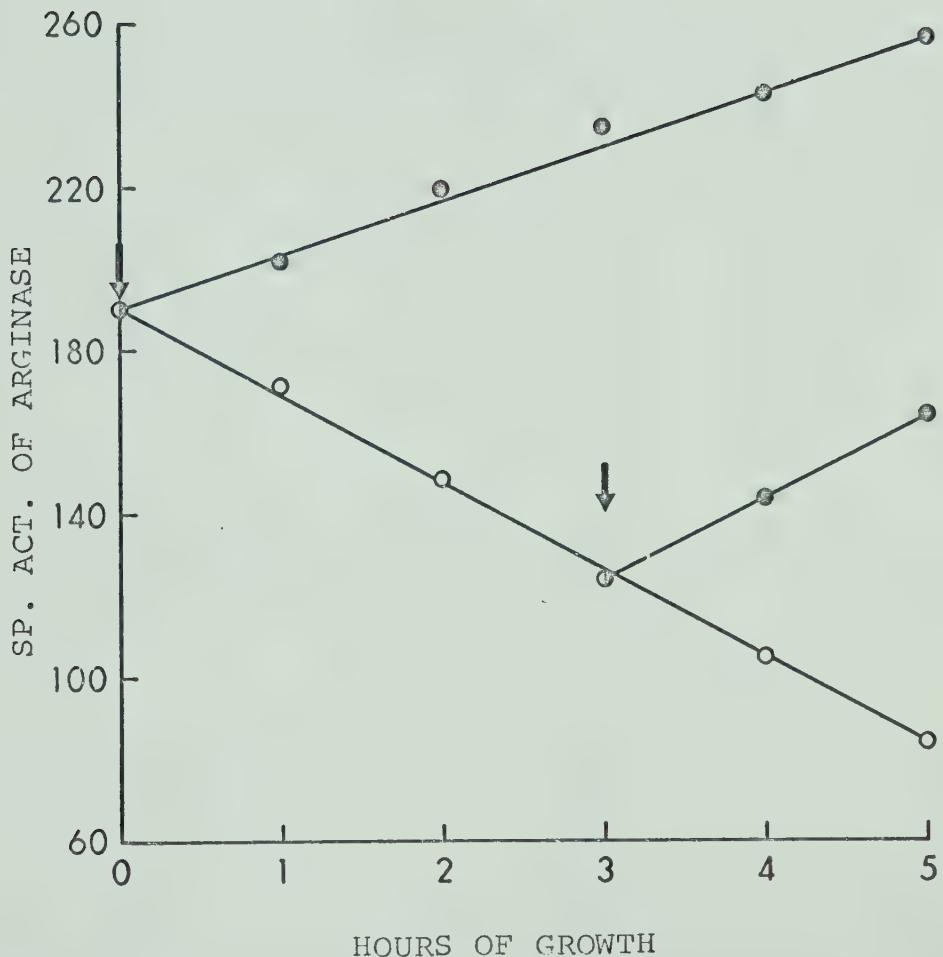
The cells were initially grown in basic culture medium supplemented with  $2 \times 10^{-2}$ M L-arginine for 4 hours at 30°C before being inoculated into a fresh basic culture medium supplemented with  $2 \times 10^{-2}$ M urea.

The arrows indicate addition of cycloheximide (10  $\mu$ g/ml of culture medium) to the culture flasks.

○ - ○ : arginase activity of cells grown in basic culture medium +  $2 \times 10^{-2}$ M urea.

○ - ● : arginase activity of cells grown in basic culture medium +  $2 \times 10^{-2}$ M urea + 10  $\mu$ g/ml cycloheximide.

Data are average values obtained from four separate experiments.







## FIGURE 7

*THE EFFECT OF CYCLOHEXIMIDE ON THE ACTIVITY OF ARGINASE OF CELLS INITIALLY CULTURED IN THE PRESENCE OF UREA*

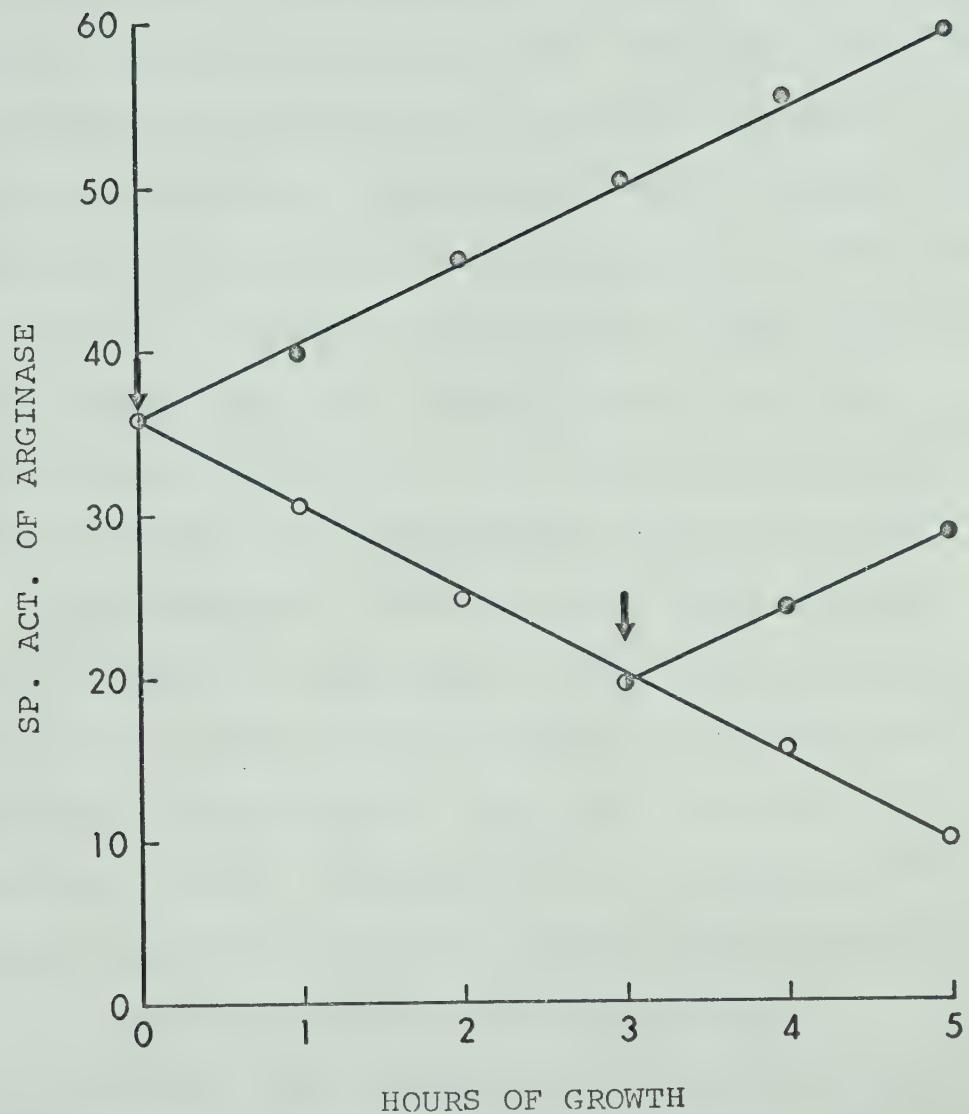
The cells were initially grown in basic culture medium supplemented with  $2 \times 10^{-2}$ M urea for 5 hours at 30°C before being inoculated into a fresh basic culture medium supplemented with  $2 \times 10^{-2}$ M urea.

The arrows indicate additions of cycloheximide (10  $\mu$ g/ml of culture medium) to the culture flasks.

○ - ○ : arginase activity of cells grown in basic culture medium +  $2 \times 10^{-2}$ M urea.

● - ● : arginase activity of cells grown in basic culture medium +  $2 \times 10^{-2}$ M urea + 10  $\mu$ g/ml cycloheximide.

Data are average values obtained from four separate experiments.





present regulation of arginase activity also appeared to require protein synthesis, it was of some interest to determine whether a similar situation existed.

Cell-free extracts prepared from cells cultured in the presence of arginine and urea were subjected to gel filtration using a column of Sephadex G-200. Care was taken in separate elutions to maintain constant flow rates and identical sample volumes. Reproducibility of elution volumes was substantiated by repeated chromatography of authentic protein markers. Variations encountered in such elutions were never greater than 5%. From Figures 8a and 8b, it is clear that samples of arginase, although differing markedly in specific activity, had essentially the same elution volumes. This identical chromatographic behavior was maintained in separate experiments where flow rates were decreased. In no case was the elution of arginase from arginine-grown cells different from that shown by the enzyme from urea-grown cells. Another similarity between such preparations was an approximate 30-fold purification of the enzyme as a result of Sephadex G-200 treatment.

The results of these studies are, therefore, distinctly different from the findings of Messenguy and Wiame (1969) where the repressed OTC enzyme had a molecular weight considerably higher than that of the normal enzyme.

*Studies of other enzymes related to the Krebs-Henseleit Cycle*

At least three other enzymes related to the Krebs-





## FIGURE 8

## GEL FILTRATION OF YEAST ARGINASE ON SEPHADEX G-200

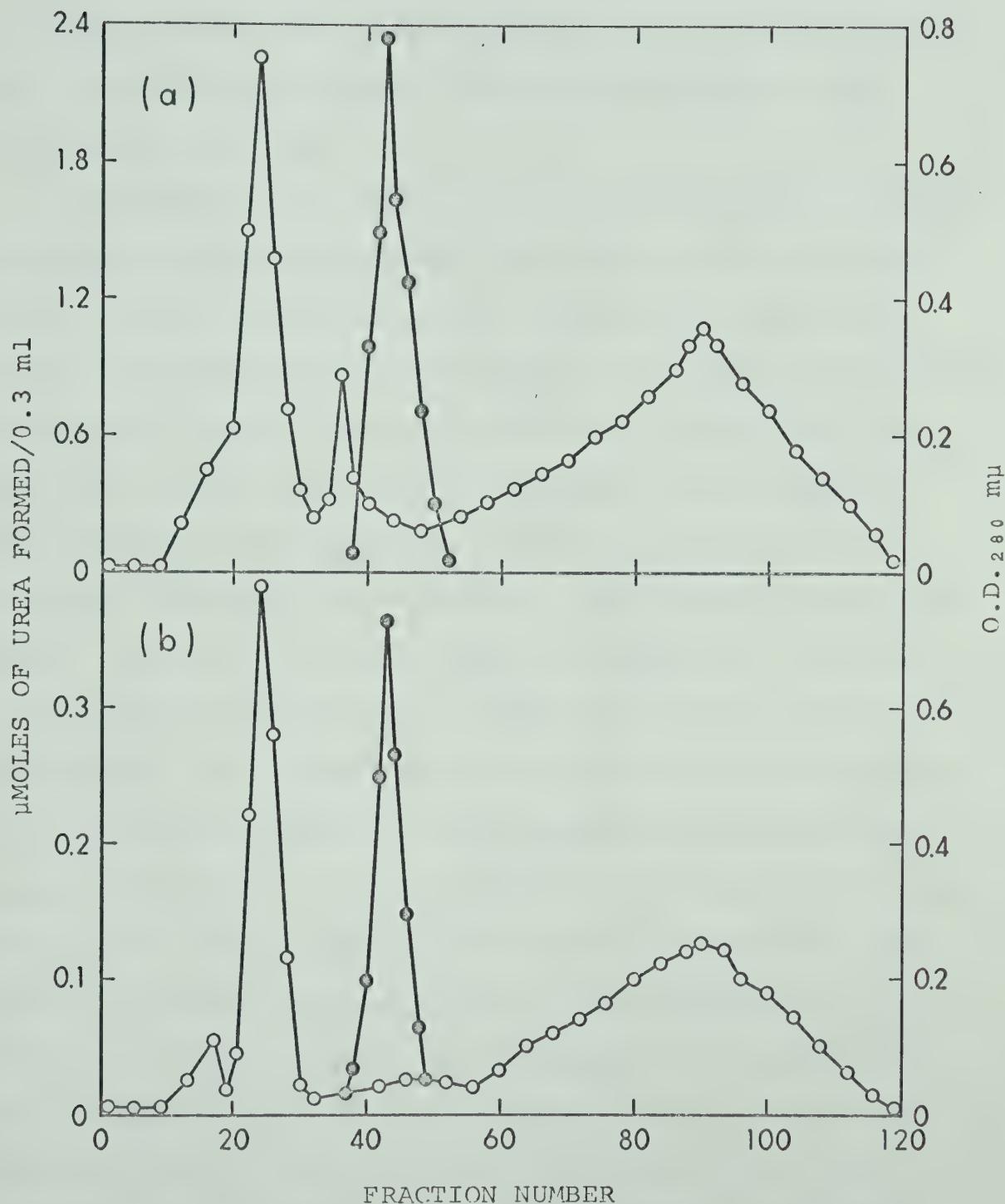
- a. The cell-free extract was prepared from yeast cells cultured for 4 hours at 30°C in basic culture medium +  $2 \times 10^{-2}$  M L-arginine.
- b. The cell-free extract was prepared from yeast cells cultured for 4 hours at 30°C in basic culture medium +  $2 \times 10^{-2}$  M urea.

Samples (0.5 ml) of the cell-free extracts, containing 8-10 mg protein, were applied to the column and eluted with 0.01 M potassium phosphate buffer (pH 7.5) at a constant flow rate of 20 ml/hr. Fractions of 3 ml were collected.

○ - ○ : O.D.280

● - ● : urea formation ( $\mu$ moles)

Maximum specific activities of arginase after gel filtration were 960 in (a) and 105 in (b), based on the averages of four separate experiments. The initial specific activities were 31 in (a) and 3.5 in (b).





Henseleit cycle, besides arginase, were examined in extracts of yeast cells. These were ornithine transcarbamylase,  $\delta$ -ornithine transaminase, and argininosuccinate lyase. In all cases the specific activities of these enzyme in cell-free extracts were compared after growth under various conditions (Table XII).

A repression of ornithine transcarbamylase by arginine was demonstrated and confirms the earlier work of Bechet *et al.* (1962); Bechet and Wiame (1965); and Middelhoven (1969). When cultured in the absence of nitrogen compounds, the specific enzyme activity of OTC was almost three times the value found in the arginine culture. This, however, when compared to the specific activity of the initial inoculum, was still low, suggesting some repression of the enzyme. Urea, on the other hand, increased the levels of ornithine transcarbamylase to some extent.  $\delta$ -Ornithine transaminase, the enzyme involved in the catabolic pathway for L-ornithine, appeared to be induced by L-arginine and repressed by urea. In the absence of a nitrogen source the transaminase levels remained essentially unaltered. The levels of argininosuccinate lyase were similar when the cells were grown in either the presence of L-arginine or urea. Under these conditions, however, the specific activities were increased about 3-fold from that of the initial inoculum. A slight decline in argininosuccinate lyase activity was observed when the cells were subjected to nitrogen starvation. On the basis of the data in Table XII



it can be concluded that the two anabolic enzymes, ornithine transcarbamylase and argininosuccinate lyase, are not induced or repressed co-ordinately. The two catabolic enzymes, arginase and  $\delta$ -ornithine transaminase, on the other hand, respond similarly to the presence of arginine and urea. Conceivably arginine is degraded in *Saccharomyces cerevisiae* by a pathway involving arginase and ornithine transaminase. In this connection both enzymes are induced by arginine and repressed by urea.

Urease activity, surprisingly, could not be detected in this organism, either in crude extracts prepared by disrupting the cells sonically or in partially purified preparations after Sephadex G-25 treatment. Attempts to assay this enzyme in acetone-dried powders were also unsuccessful. Such extracts did not contain an inhibitor of urease activity since the activities of commercial urease preparations (Sigma Chemical Company) were unaffected by the addition to them of freshly prepared yeast extracts.

Another catabolic enzyme, the arginine deiminase, which produces citrulline and ammonia from arginine, was also not detected in the present yeast extracts. This enzyme was, however, reported in yeast cells by Roche and Lacombe (1952) who found the activity in acetone powders to be optimal at pH 5.8-7.0 after activation with  $\text{Co}^{++}$ . In the present study, yeast cells were acetone-dried, extracted and purified by ethanol precipitation according to Oginsky (1960). The reaction system contained 0.1 M L-arginine in



TABLE XII

SPECIFIC ACTIVITIES OF OTC, ASL, OTA, ARGINASE AND UREASE IN YEAST, GROWN IN BASIC CULTURE MEDIUM SUPPLEMENTED WITH DIFFERENT NITROGEN SOURCES

N-source added after cells cultured in basic medium + 10 ml of amino acids per litre for 24 hours	Hours of Incubation	OTC Specific Activity	ASL Specific Activity	OTA Specific Activity	Arginase Specific Activity
Initial inoculum	0	14.5	0.28	2.3	28.8
Culture in the absence of N-source	5	6.2	0.265	2.8	60.0
L-arginine ( $2 \times 10^{-2} M$ )	5	2.2	0.63	4.2	130.0
Urea ( $2 \times 10^{-2} M$ )	5	19.5	0.70	0.9	14.4

The following abbreviations are used: OTC = ornithine transcarbamylase

ASL = argininosuccinate lyase

OTA = ornithine transaminase

Specific activity of urease was not detectable in every case under the above culture conditions. The data are average values obtained from determinations made from 3 separate experiments.



0.2 M phosphate buffer (pH 6.5),  $\text{Co}^{++}$  and  $\text{Mn}^{++}$  being used as activators. The possible production of citrulline was examined by colorimetric assay as stated on Page 34, and after electrophoresis on silica gel G thin-layer plates (Eastman Kodak, Rochester, N.Y., U.S.A.) using 0.05 M sodium borate/0.05 M succinate buffer at pH 7.0, followed by a ninhydrin spray. All of these results were negative. It is concluded, therefore, that arginine deiminase, although commonly found in some bacteria (Ojinsky and Gehrig, 1952) and green algae (Shafer and Thompson, 1968) was not present in the *Saccharomyces cerevisiae* cells used in the present work.

*Levels of free amino acids,  $\text{NH}_4^+$  and urea in yeast extracts and media after growth*

a. *Culture medium*

After 5 hours of incubation, the culture medium was examined for the levels of amino acids present. Urea, citrulline and arginine were assayed colorimetrically as stated in the Materials and Methods. The remaining compounds, including arginine, were measured by a Beckman Amino Acid analyzer, Model 121. The results are presented in Table XIII. No amino acids were found in the basic culture medium after 5 hours of growth, except for a trace amounts of ammonia. In the arginine-supplemented culture, 14 mM L-arginine remained in the medium, indicating a 30% uptake of this amino acid by the cells during 5 hours of growth.



TABLE XIII

LEVELS OF NITROGENOUS COMPOUNDS IN THE CULTURE MEDIA

BEFORE AND AFTER 5 HOURS

(Data are expressed as mM concentrations)

Culture conditions	Ammonia	Arginine	Urea	Ornithine
<b>Basic medium:</b>				
Before growth	0	0	0	0
After growth	<0.01	0	0	0
<b>Arginine medium:</b>				
Before growth	0	20	0	0
After growth	<0.01	14	0	2.15
<b>Urea culture:</b>				
Before growth	0	0	20	0
After growth	8.60	0	15	<0.01

The data are average values obtained from determinations made in 4 separate experiments.



At the same time, 2.15 mM ornithine was detected in this medium, an amount equivalent to 1600  $\mu$ moles of ornithine/g.d. wt. of cells. Ornithine was, therefore, excreted by the cells and accumulated in the medium. Ammonia, again, was detected in trace amounts only. In the urea supplemented medium, 15 mM urea was detected after 5 hours of incubation. This indicates a net uptake of 25% of the supplied urea. In addition, 8.6 mM ammonia, equivalent to 11800  $\mu$ moles/gm dry weight accumulated in the medium.

b. *Cell-free extracts*

The levels of amino acids and  $\text{NH}_4^+$ , present in the cell-free extracts, were assessed as described in Materials and Methods and in Section A of this part. Yeast extracts, prepared from cells grown in the basic culture medium, showed a generally low level of free amino acids as compared to extracts of cells grown in arginine-rich or urea-rich media (Table XIV). This is to be expected as the yeast, undergoing active growth, will require a large amount of amino acids for incorporation into proteins and other cellular constituents.

Extracts of cells grown in the basic culture medium contained the lowest level of total amino acids. The principal component of the amino acid pool of such cells was glutamic acid, accompanied by significant levels of serine, glycine and alanine. Ammonium ions were also detected without difficulty. Under the conditions of assay only trace amounts of arginine (less than 0.1  $\mu$ mole/g.d.wt.



TABLE XIV

LEVELS OF FREE AMINO ACIDS IN CELL-FREE EXTRACTS FROM  
 DIFFERENT CULTURE MEDIA  
 (after 5 hours incubation†)

N-Compounds	Basic culture μmoles/g.d.wt.	Arginine culture μmoles/g.d.wt.	Urea culture μmoles/g.d.wt.
lysine	2.6	33.20	22.5
histidine	<0.1	<0.1	n.d.
NH <sup>+</sup>	10	32.0	41.6
arginine	<0.1	111.2	10.3
citrulline	n.d.	n.d.	n.d.
ornithine	n.d.	n.d.	n.d.
urea	n.d.	50.0	n.d.
aspartic acid	2.8	6.3	15.3
threonine	5.6	7.0	22.7
serine	12.6	8.07	49.0
glutamic acid	18.0	61.6	420.0
proline	0.80	4.1	8.20
glycine	11.80	8.6	23.6
alanine	10.90	11.0	48.0
half-cystine	n.d.	n.d.	n.d.
valine	6.7	6.0	11.5
methionine	n.d.	n.d.	n.d.
isoleucine	0.7	1.50	2.0
leucine	0.7	1.65	1.7
tyrosine	n.d.	n.d.	n.d.
phenylalanine	n.d.	n.d.	n.d.
Totals	83.20	342.2	676.4

† Amount of cells harvested after 5 hours of growth from the same amount of inoculum were: Basic culture - 0.096 gm.d.wt.; L-arginine culture - 0.124 gm.d.wt.; Urea culture - 0.187 gm.d.wt. Data were taken as the average of at least 3 separate experiments. n.d. - not detected.



of cells) were detected in these extracts. In contrast, extracts of cells grown on the arginine-supplemented medium contained high levels of this amino acid. Large increases were also evidence for lysine, glutamic acid, urea and ammonia. In urea-supplemented cultures, the levels of the free amino acids were again considerably increased. Surprisingly, urea was not detected in these cell-free extracts despite repeated attempts to measure this compound. Growth in the presence of urea greatly increased the levels of glutamic acid, arginine, ammonia, alanine and aspartic acid.

The results of these analyses, therefore, support the earlier enzyme studies by suggesting that arginine and urea are both actively metabolized by the cells. This is evident not only from a consideration of the changes caused in the free amino pools and changes in the components of the growth medium but also from the amounts of growth observed when these supplements were made (Table XIV).

#### *Some general properties of yeast arginase*

##### *1. The effect of enzyme concentration on reaction rates*

The standard colorimetric assay procedure was used to measure the effect of protein concentration on arginase activity. The results (Figure 9) show that the reaction velocities for arginase when expressed as a function of protein concentration were linear. This linear relationship was found up to protein levels of 250  $\mu$ g. In subsequent





## FIGURE 9

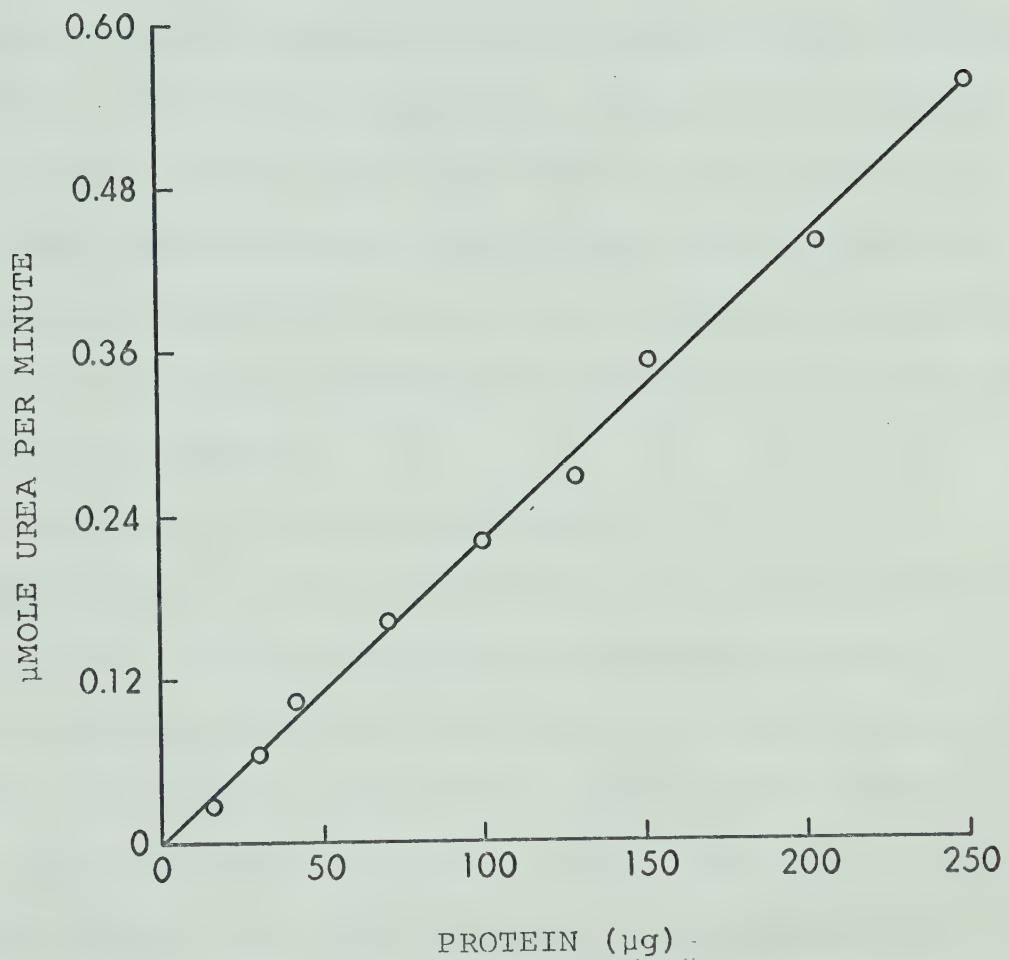
*ARGINASE ACTIVITY AS A FUNCTION OF PROTEIN CONCENTRATION*

Reaction systems contained the following in a total volume of 0.5 ml:

10  $\mu$ moles of manganese maleate (pH 7.0); 140  $\mu$ moles of L-arginine and varying amounts of enzyme preparation (Sephadex G-25 treated) as indicated.

The enzyme was preincubated at 30°C for 30 minutes with manganese maleate buffer only and then incubated for 10 minutes with the addition of L-arginine. Final pH 9.2.

The data are average values from three separate experiments.





work, samples of enzyme containing 100 to 200  $\mu$ g of protein were routinely used for assay of arginase activity.

## 2. *The effects of incubation time on product formation*

Samples taken after different periods of incubation (Figure 10) show that the rate of product formation was linear for the first 10 minutes only. This is in agreement with earlier work by Vanslyke and Archibald (1946); Gilboe and Williams (1956) who found that a linear relationship between enzyme activity and time did not exist after 10 minutes with liver arginase but differs from a report by Splittstoesser (1969) who found that in pumpkin cotyledons, the amount of arginine hydrolyzed with time, was linear for as long as 120 minutes.

## 3. *Effects of pH on arginase activity*

From Figure 11, it is seen that yeast arginase has a pH optimum of 9.2. This pH optimum was determined when manganese maleate was used as buffer over a pH range of 7.0 and 10.5. This result, therefore, agrees with similar studies on the enzyme from bovine liver (Bach and Killip, 1960) but differs from the optimum of 9.5 reported by Carlisky *et al.* (1968) for amphibian kidney, and 10.0 for the enzyme from insect fat body (Reddy and Campbell, 1969). Middelhoven (1969), Vaidyanathan and Giri (1953), Muszynska and Reifer (1968) and many other workers found that the pH optima of yeast arginase varied depending on the presence of various metallic ions which acted as activators.





FIGURE 10

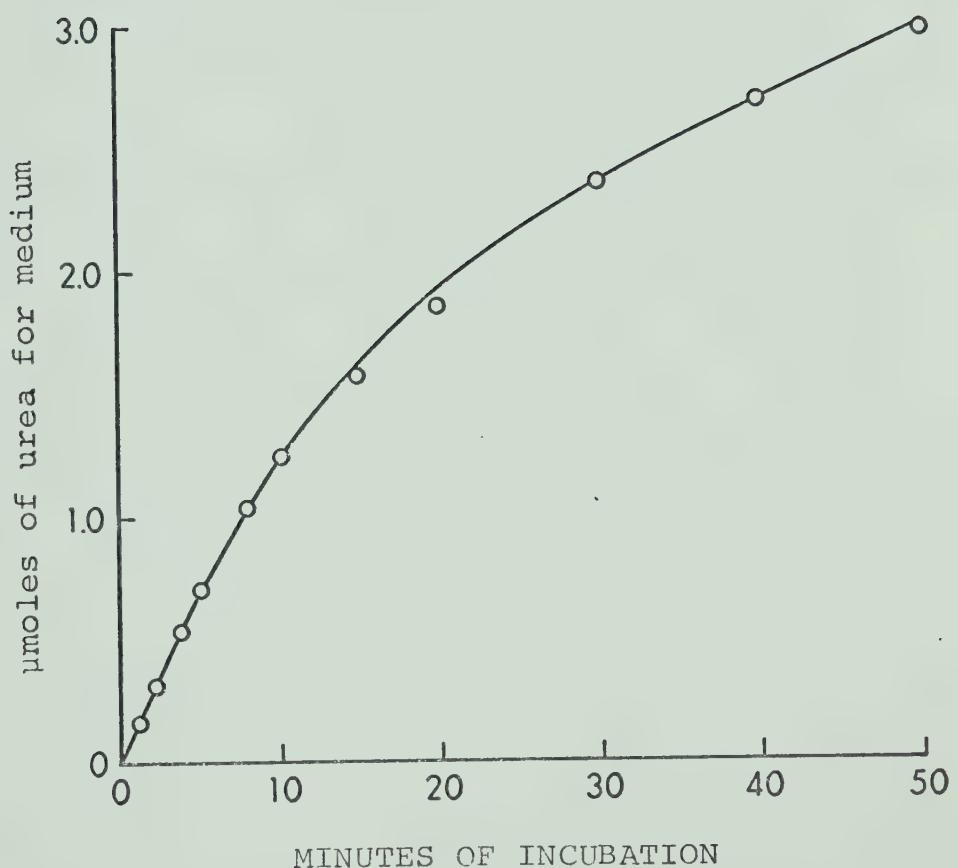
*THE RELATIONSHIP BETWEEN ARGINASE ACTIVITY AND REACTION TIME*

Reaction mixtures containing the following in a total volume of 0.5 ml:

10  $\mu$ moles of manganese maleate (pH 7.0); 140  $\mu$ moles of L-arginine (pH 9.2); and 150-200  $\mu$ g of protein from the cell-free extract after Sephadex G-25 treatment.

After 30 minutes of preincubation with manganese maleate the reaction systems were incubated at 30°C for the various times indicated. The assay was as outlined in the Materials and Methods section.

The data are average values from three separate experiments.







## FIGURE 11

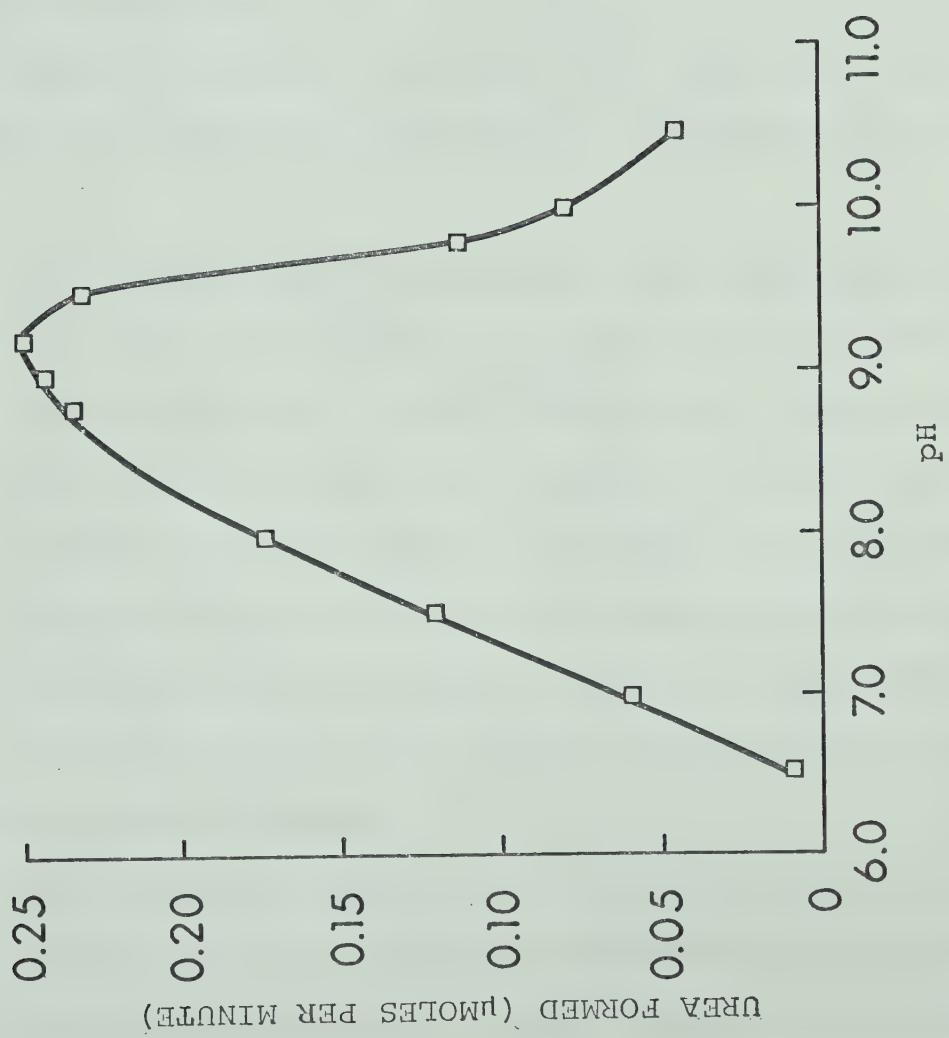
## THE EFFECT OF pH ON ARGINASE ACTIVITY

Reaction mixtures containing the following in a total volume of 0.5 ml:

10  $\mu$ moles of manganese maleate (pH 7.0): 140  $\mu$ moles of L-arginine, and Sephadex G-25 treated cell-free extract (approximately 125  $\mu$ g protein).

The enzyme was preincubated at 30°C for 30 minutes with manganese maleate buffer and then incubated for 10 minutes in the presence of substrate adjusted to the pH values indicated.

The data are average values from three separate experiments.





#### 4. Effects of metallic ions on arginase activity

Yeast arginase, in agreement with other work, was activated by divalent metallic ions. Table XV indicates that manganese in a concentration of 20  $\mu$ moles/ml reaction system gave the most effective activation of the enzyme. Cobalt and ferrous ions also activate arginase to some extent, but not as effectively as with manganese.

#### 5. Michaelis constants

The apparent Michaelis constants for arginase were determined from Lineweaver-Burk plots (Lineweaver and Burk, 1934).

- a. With sodium glycinate as buffer and manganese ions + Tris-HCl as activator, the calculated apparent  $K_m$  for arginase was 12.5 mM (Figure 12). Mora *et al.* (1966) obtained a  $K_m$  for *Neurospora crassa* arginase of 100-200 mM and 20-40 mM for rat liver arginase. Thus the apparent affinity for arginine displayed by the yeast enzyme is about 10 times greater than the *Neurospora* enzyme and slightly lower than that of the rat liver arginase.
- b. With potassium phosphate as buffer and manganese ions as activator, the calculated apparent  $K_m$  for arginase was 250 mM (Figure 13). When compared to the sodium glycinate buffer system indicated above, the apparent affinity for the substrate was, therefore, approximately 20 times lower than in the earlier experiment. This suggests that arginase is inhibited



TABLE XV

## EFFECT OF METALLIC IONS ON THE ACTIVITY OF YEAST ARGINASE

Metallic salt added	Final concentration μmoles/ml	Buffer system	pH	Specific Activity
None		Sodium glycinate	7.0	34
None		Sodium maleate	7.0	40
Mn Maleate	4	Mn maleate	7.0	58
"	10	"	7.0	70
"	16	"	7.0	106
"	20	"	7.0	132
CoCl <sub>2</sub>	20	Sodium glycinate	7.0	65
FeCl <sub>2</sub>	20	Sodium glycinate	7.0	68

In all cases, partially purified enzyme (containing 120 μgm protein) was used. When sodium glycinate was used as the buffer, the reaction system contained 4 μmoles of Mn<sup>++</sup> Tris-HCl (pH 7.0) as activator and was activated at 50°C for 15 minutes. Then 100 μmoles of L-arginine (pH 9.2) and 10 μmoles of the buffer were added and incubated at 30°C for 10 minutes. When Mn maleate was used as the buffer, the reaction system contained 10 μmoles of the buffer and was activated at 30°C for 30 minutes. Then 100 μmoles of L-arginine (pH 9.2) was added and was incubated at 30°C for 10 minutes. The data represents average values obtained from 3 separate experiments.





FIGURE 12

*ARGINASE ACTIVITY AS A FUNCTION OF SUBSTRATE CONCENTRATION*

The reaction systems contained the following in a total volume of 0.5 ml:

4  $\mu$ moles of manganese Tris HCl buffer mixture (pH 7.5);  
4  $\mu$ moles sodium glycinate (pH 7.5); Sephadex G-25  
treated extract (100  $\mu$ g protein) and varying amounts  
of L-arginine as indicated. Final pH 9.2.

The enzyme was preincubated at 50°C for 15 minutes with manganese Tris-HCl and then incubated for 10 minutes at 30°C with the sodium glycinate and substrate.

The data are average values from three separate experiments.

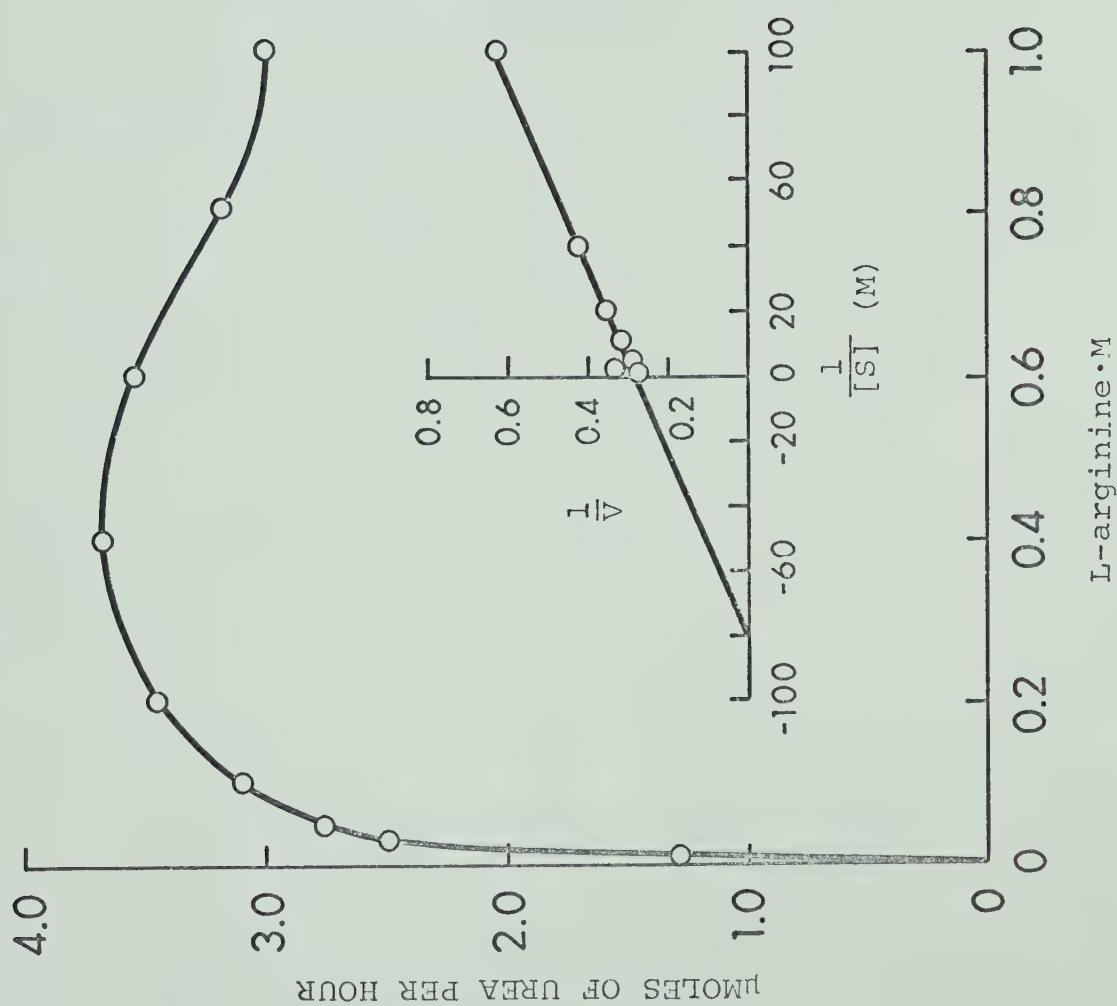






FIGURE 13

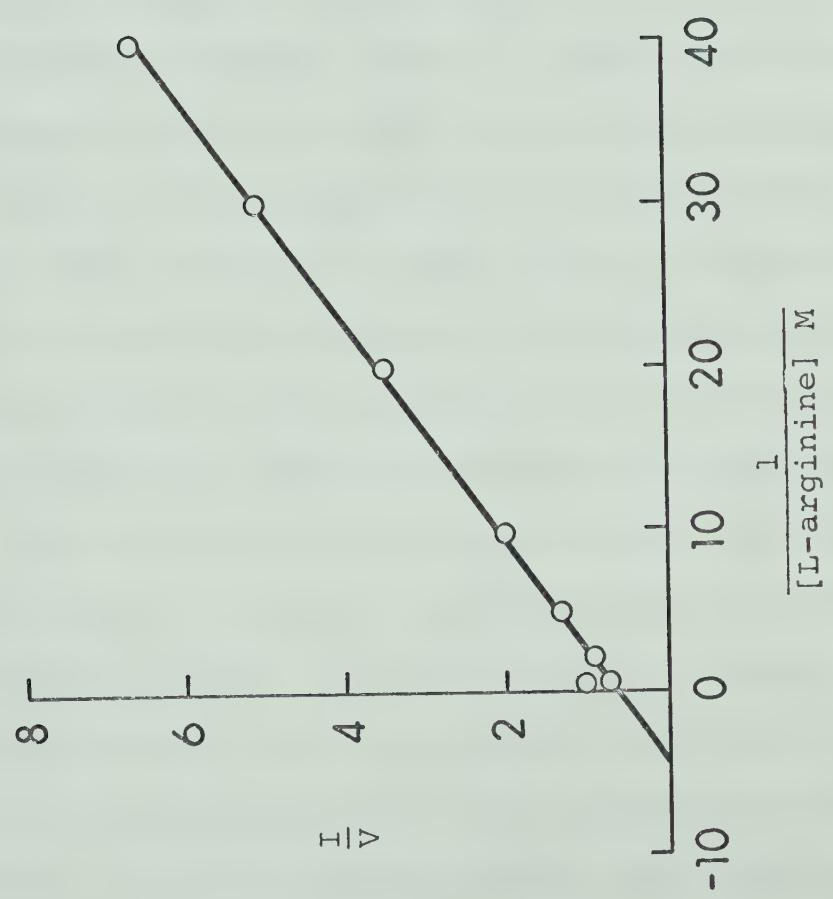
*LINEWEAVER-BURK PLOT OF INITIAL REACTION VELOCITY  
VERSUS L-ARGININE CONCENTRATION*

The reaction mixtures contained the following in a total volume of 0.5 ml:

4  $\mu$ moles of potassium phosphate (pH 7.2); 10  $\mu$ moles of manganese maleate (pH 7.0); Sephadex G-25 treated extract (100  $\mu$ g protein) and varying amounts of substrate. Final pH 9.2.

The enzyme was preincubated with potassium phosphate and manganese maleate for 30 minutes at 30°C before incubation with substrate for 10 minutes at 30°C.

The data are average values from three separate experiments.





by phosphate ions to some extent. In studies of yeast arginase, Middelhoven (1969) reported that at least in its native form, yeast arginase is strongly inhibited by phosphate. Table XVI summarizes the different  $K_m$  values obtained for arginine when various amounts of L-ornithine were added to systems buffered by sodium glycinate and manganese maleate respectively.

#### 6. Inhibition of arginase by L-ornithine

It was mentioned earlier (Table X) that L-ornithine exerted a profound inhibitory effect on arginase activity. Cross (1921) was the first investigator to report this inhibition and later, Hunter and Downs (1944) extended this by studying the effect of a series of amino acids on arginase activity. They found that all  $\alpha$ - amino acids of the natural occurring L configuration inhibited arginase activity to some extent. The results of experiments to test the kinetic behavior of arginase at various concentrations of L-ornithine are shown in Figures 14 and 15. These results clearly indicate that L-ornithine is a competitive inhibitor of arginase in both potassium phosphate and sodium glycinate buffers systems. In sodium glycine buffer, the enzyme was more sensitive to L-ornithine than in the potassium phosphate system. This is reasonable since phosphate itself is an inhibitor to the enzyme (Table XVI) and this may desensitize the enzyme.





## FIGURE 14

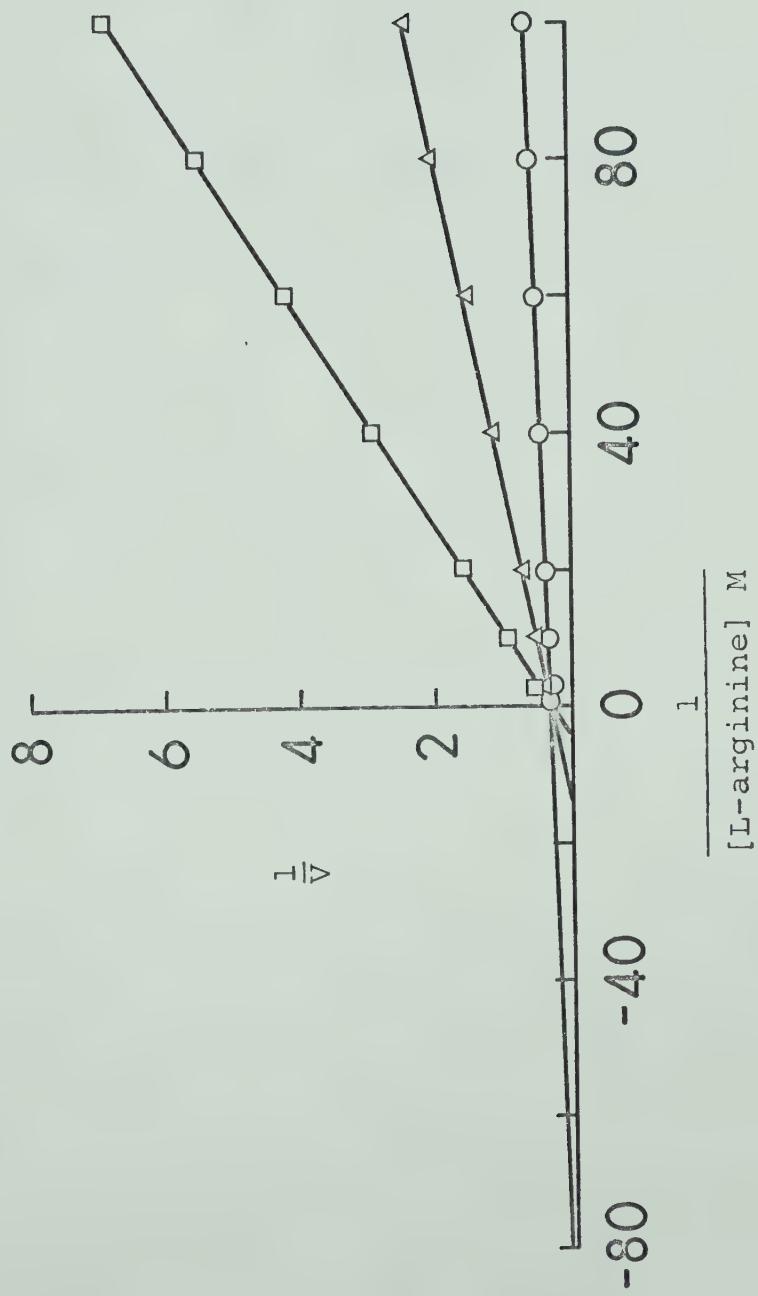
*THE INHIBITORY EFFECT OF L-ORNITHINE ON ARGINASE ACTIVITY IN  
THE PRESENCE OF SODIUM GLYCINATE*

The reaction mixtures contained the following in a total volume of 0.5 ml:

4  $\mu$ moles of manganese Tris HCl buffer (pH 7.5); 4  $\mu$ moles sodium glycinate (pH 7.5); Sephadex G-25 treated extract; and varying amounts of L-arginine as indicated. Final pH 9.2.

The enzyme was preincubated at 50°C for 15 minutes with manganese Tris HCl and then incubated for 10 minutes at 30°C with the sodium glycinate and L-arginine.

Reaction systems ( $\square - \square$ ) and ( $\Delta - \Delta$ ) contained 10  $\mu$ moles and 5  $\mu$ moles of L-ornithine respectively.







## FIGURE 15

*THE INHIBITORY EFFECT OF L-ORNITHINE ON ARGINASE ACTIVITY  
IN THE PRESENCE OF POTASSIUM PHOSPHATE BUFFER*

The reaction system contained the following in a total volume of 0.5 ml:

4  $\mu$ moles of potassium phosphate (pH 7.2); 10  $\mu$ moles of manganese maleate (pH 7.0); Sephadex G-25 treated extract (100  $\mu$ g protein) and varying amounts of substrate. The final pH was 9.2.

The enzyme was preincubated with the potassium phosphate and manganese maleate for 30 minutes at 30°C before incubation with substrate for 10 minutes at 30°C.

Reaction systems (● - ●), (□ - □), and (Δ - Δ) contained in addition 100, 50 and 25  $\mu$ moles of L-ornithine respectively.

The data are average values from three separate experiments.

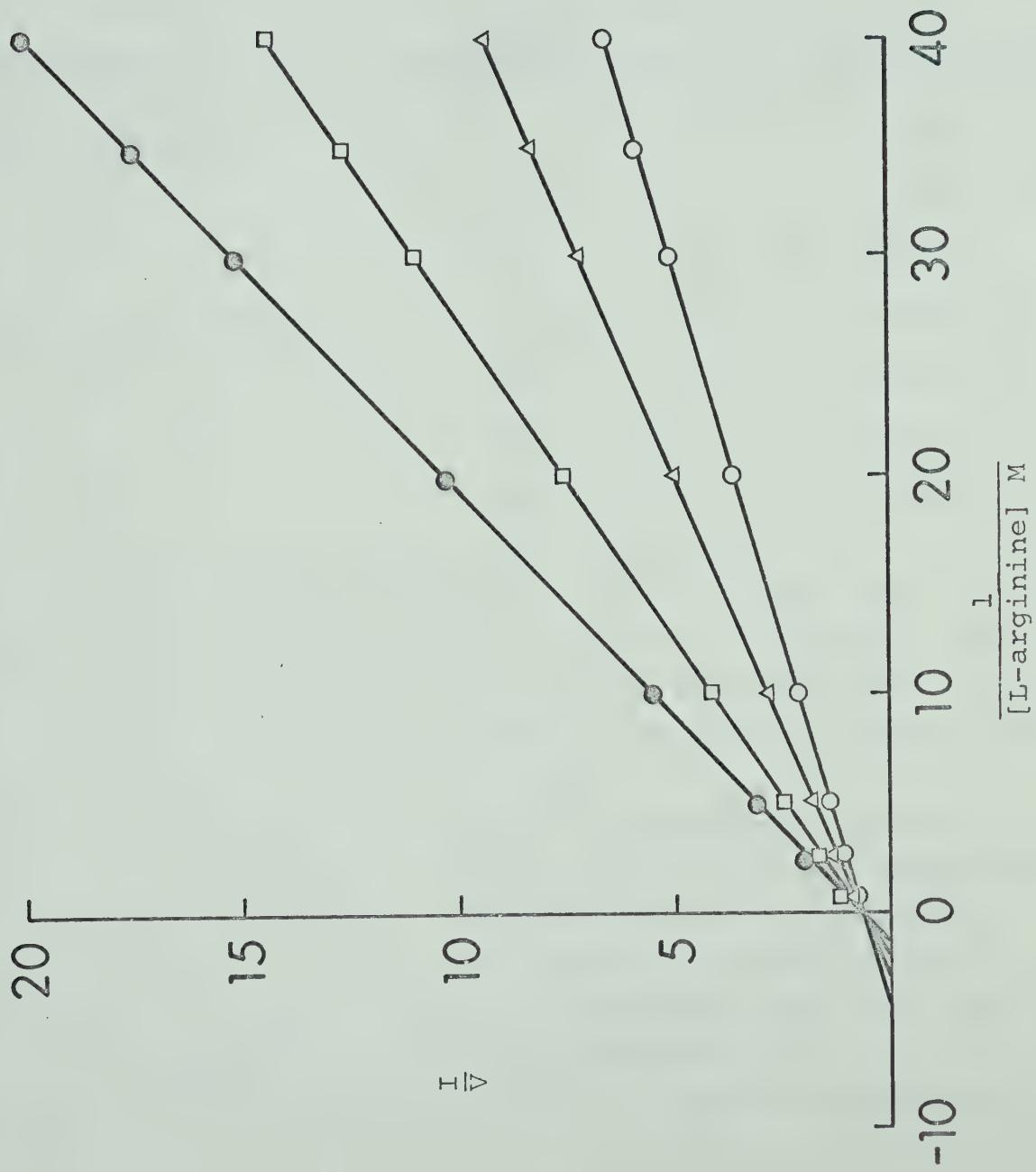




TABLE XVI

*MICHAELIS CONSTANTS FOR ARGININE IN THE PRESENCE OF  
COMPETITIVE INHIBITORS*

Buffer System	μmoles of L-ornithine added	$K_m$ for L-arginine (mM)
Sodium glycinate*	0	12.5
"	1	84.0
"	2	250.0
Potassium phosphate**	0	250.0
"	25	300.0
"	50	500.0
"	100	770.0

\* The system contained 4 μmoles of  $Mn^{++}$  Tris-HCl (pH 7.0) as activator and was incubated at 50°C for 15 minutes. Then 10 μmoles of the buffer (pH 7.0) and 100 μmoles of L-arginine (pH 9.2) were added to the reaction system, and incubated at 30°C for 10 minutes.

\*\* The system contained 2 μmoles of potassium phosphate, pH 7.0; 10 μmoles of Mn maleate, pH 7.0 and was activated at 30°C for 30 minutes. Then 100 μmoles of L-arginine (pH 9.2) was added, and was incubated at 30°C for 10 minutes. 140 μgm of partially purified enzyme was used in each system throughout the experiment. Data were taken as the average of 3 separate experiments.



### 7. Molecular weight determinations

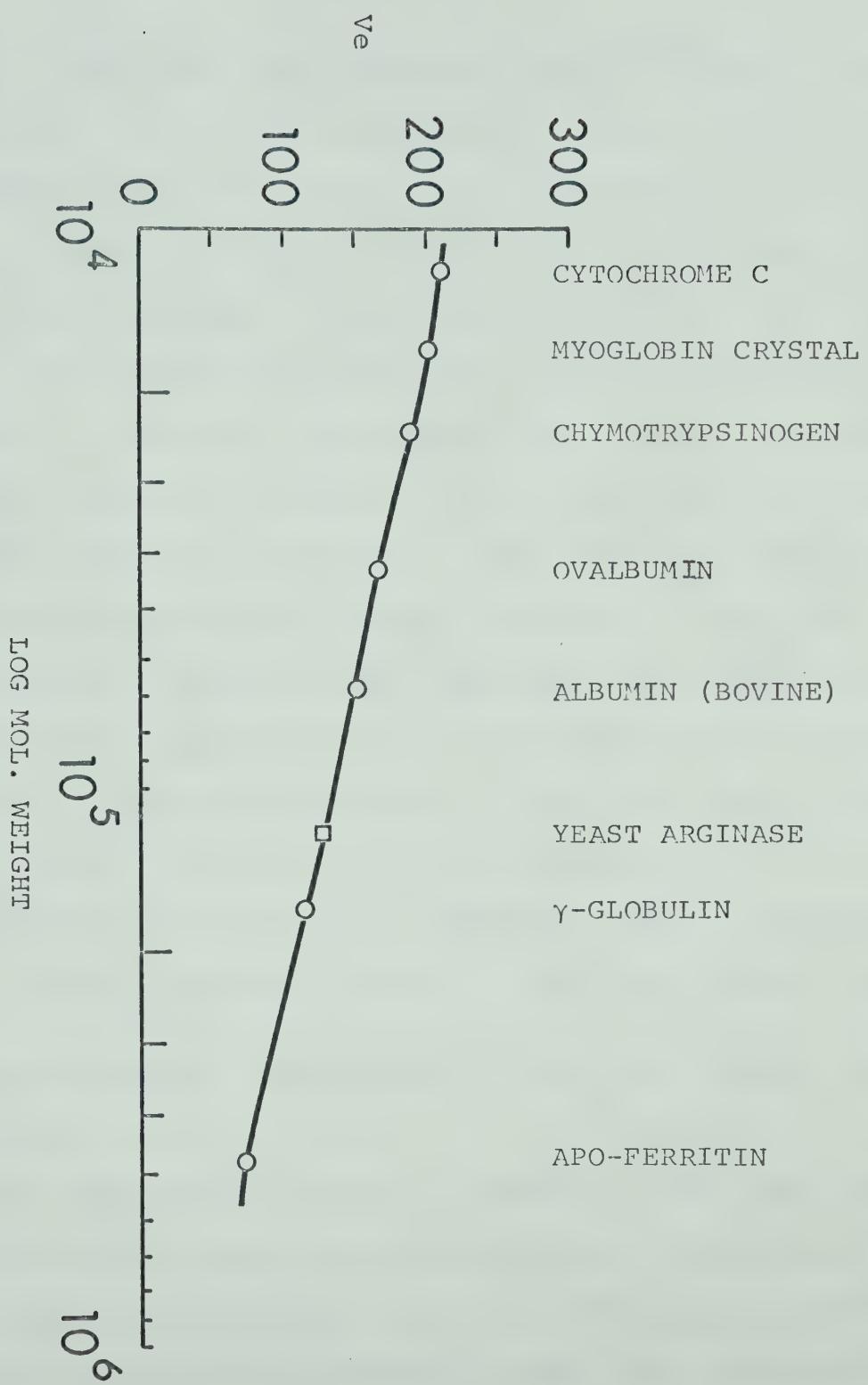
For molecular weight determination, a sample containing approximately 5 mg protein was passed through a column of Sephadex G-200 together with highly purified proteins of known molecular weight. The column effluent was assayed for yeast arginase activity and for protein as described in the Materials and Methods. Elution volumes were estimated as described by Andrews (1964) and plotted against log molecular weight for the authentic proteins applied to the column (Figure 16). On the basis of 3 separate experiments, the average molecular weight for yeast arginase was estimated to be approximately 120,000. The results confirm those of Messenguy and Wiame (1969) who estimated the molecular weight of yeast arginase to be  $114,000 \pm 7,000$ .

FIGURE 16

*GEL FILTRATION OF YEAST ARGINASE AND MARKER PROTEINS*

Samples of yeast arginase and authentic proteins were applied to columns of Sephadex G-200 and eluted as described in the Materials and Methods.

The elution volumes are average values from four separate experiments.





## DISCUSSION

The rapid and extensive growth of *Saccharomyces cerevisiae* cells in urea supplemented culture medium (Figure 4A) suggests that this compound is utilized as a sole nitrogen source. The function of arginase is to produce urea by the splitting of arginine. When urea is abundant in the medium it will be economical for the cell to stop production of this substance. There are two possible ways the cell can control formation of a given product: (1) it may change the number of enzyme molecules available to catalyze the production or (2) it may change their catalytic activity. The former can be achieved by repression and the latter by feedback inhibition. Both controls appear to exist in *Saccharomyces cerevisiae* in the case of arginine metabolism. From this study, it was found that the arginase activity of yeast cultures was finely controlled by the presence of substrate and end products. The following discussion concerns itself with these aspects.

*Changes of Arginase Activity as a Result of Induction and Repression*

The experimental results in Table III show that arginase activity varies depending on the nitrogen contents of the culture media. L-arginine, at a concentration of  $2 \times 10^{-2} M$ , gave the highest enzyme activity, while urea and ammonia at the same concentration gave the lowest levels. Intermediate cases existed at different concentrations of the above nitrogenous compounds, as well as mixtures of the above and



the presence of other nitrogenous compounds such as ornithine and citrulline. These results indicate that in cells, subjected to a changing environment, the levels of arginase are scrupulously controlled. Support for this is apparent from the results in Table IV which show these changes were reversible on transfer of the cells to suitable media. Furthermore, Table V shows that such a change depends on the period of growth. The action of arginine on arginase activity is clearly an induction effect. When the cells were grown in urea-rich media and then transferred to an arginine-rich medium, this enzyme is synthesized extensively. A time course study of this derepression (Table V) revealed that there was an explosive preferential synthesis of the enzyme which restored the normal level in a fraction of a generation. This continuous 'derepressed' synthesis of the enzyme will be possible throughout the growth time and will occur at a slow and steady rate. It is clear from these and published observations (Middelhoven, 1964, 1969) that cells have a reserve capacity to synthesize various enzymes at rates far exceeding those associated with ordinary steady-state growth. This capacity is of prime importance in the adaptation of such cells to a changing environment.

The exact nature of arginase induction by its substrate is, however, still not fully understood. The genetic analysis of induction of nitrate reductase in *Aspergillus nidulans* (Pateman and Cove, 1967) suggests that nitrate interacts with the product of a regulatory gene to form an active complex



which binds to the operator site and permits initiation of operon transcription. Another model was proposed by Gilbert and Müller-Hill (1967). They reported that the product of the regulatory gene of *E. coli* lac operon is prevented from binding to the operator site by an inducer, and transcription is initiated in its absence. The control system in yeast may fall into one of these two categories. Future work involving inhibitors which help to block peptide-bond formation or mRNA synthesis may elucidate this problem.

The fact that arginase activities from arginine-cultured and urea-cultured cells were purified to the same extent by Sephadex G-25 and G-200 treatment (Table VI and Figures 8A and 8B) strongly supports the contention that induction and repression occur as a result of changes in enzyme synthesis *de novo*. The effect of arginine and urea on arginase levels could not be removed as a result of gel filtration. The results in Table VII and VIII further indicate that the change in arginase level could be ascribed to changes in the rate of enzyme synthesis and were not simply a result of increased or decreased protein content.

Cycloheximide (sometimes used under the trade name of actidione) has been used extensively as an inhibitor of protein synthesis in living cells. It does not affect amino acid activation or synthesis of aminoacyl-tRNA (Siegel and Sisler, 1964), but is considered to inhibit (i) the transfer reaction in peptide-bond formation (Grollman, 1966), and (ii) initiation of polypeptide chain formation in wheat



embryos (Marcus and Feeley, 1966). Godchaux *et al.* (1967) in their work with rabbit reticulocytes, found that cycloheximide had a differential effect on initiation and extension of polypeptide chains. The latter process being more susceptible to initiation at very low concentrations. Ennis and Lubin (1964) reported that this antibiotic did not inhibit protein synthesis in cell-free extracts from *E. coli*. All these suggestions indicate that cycloheximide is useful in the study of control mechanisms at the gene level where, according to Jacob and Monod (1961), genetic determinants, called regulator and operator genes, control the rate of protein synthesis through the intermediacy of cytoplasmic components or repressors. Ample evidence is now available to prove that the repressor molecule is a polymeric protein (Riggs and Bourgeois, 1968; Riggs *et al.*, 1968).

The observed growth of *Saccharomyces cerevisiae* when cycloheximide was applied to the culture media can be explained on the basis of the above discussion as the absorbance of the culture decreased slowly and progressively with time (Figure 5). Immediately following the application of cycloheximide to the urea supplemented culture medium, repression was stopped and the specific activity of arginase began to increase within 30 minutes. Such increase of enzyme activity was slow and gradual (Figures 6 and 7). This effect strongly suggests that the decrease of enzyme activity in urea-culture medium is due to repression. Urea probably serves as a specific metabolite capable of activating the



repressor, the rate of repression being related to the level of the repressor, a situation in agreement with the suggestion of Sadler and Novick (1965).

Induction of arginase in the presence of arginine would, therefore, be associated with inactivation of the repressor. Cycloheximide inhibits the synthesis of protein, hence inhibits formation of both the repressor and arginase. However, this effect will be a differential one. For example, since there are only limited molecules of the repressor synthesized per genome in each generation, (Sadler and Novick, 1965; Weismeyer, 1966), it is logical to assume that synthesis of the repressor will be terminated well before arginase synthesis stops. At the same time, the synthesis of other proteins in the cell will stop gradually resulting in an apparent increase in the specific activity of arginase. The specific activity of arginase will, therefore, be maintained and will subsequently increase in this manner until degradation of the enzyme *in vivo* becomes obvious. The same situation applies if cycloheximide is added at different intervals to the culture media (Figures 6 and 7).

#### *Other Enzymes Related to the Krebs-Henseleit Cycle*

Three other enzymes catalyzing reactions of the urea cycle have been demonstrated in the present work using cell-free preparations of *S. cerevisiae*. These are ornithine transcarbamylase, argininosuccinate lyase and ornithine transaminase. As ornithine transcarbamylase is strongly repressed by arginine (DeDeken, 1962; Bechet and Wiame, 1965),



operation of the cycle can be regulated at this point. According to DeDeken (1962), 20 fold variations in the specific activity of this enzyme can occur in the presence or absence of arginine. In the present studies, this enzyme was repressed 7 fold in the presence of  $2 \times 10^{-2} M$  arginine. In contrast, enzyme activity appeared to be induced to a smaller extent, when the cells were grown in urea supplemented media (Table XII).

Although the levels of argininosuccinate lyase were slightly affected by the presence of arginine (Table XII), such variations are probably insufficient to be of physiological importance in regulation of the cycle. Similar conclusions have been reached by Middelhoven (1969) on the basis of experiments conducted with *S. cerevisiae* cultured on different media. Further support for this comes from the finding (Table XII) that the enzyme activities were found to be very similar in cells grown in arginine-rich and urea-rich culture media.

Bechet *et al.* (1962) reported that in *S. cerevisiae*, all the enzymes of the biosynthetic pathway leading to arginine are pleiotropically repressed by this amino acid. This pleiotropicity is not expressed to the same extent by all of the enzymes of this pathway. In the case of argininosuccinate lyase, arginine has less influence than it exerts on ornithine transcarbamylase. The latter occupies an important position in the cycle (Figure 1, page 7) as it functions at a branching point, at which ornithine may be diverted for



arginine synthesis or alternatively may be degraded via  $\Delta_1$ -pyrroline carboxylate to glutamate. As mentioned in the Introduction, it is of interest to compare the different levels of control which exist in various microorganisms. When such comparisons are made, it is clear that considerable variation exists. For example, in *Chlamydomonas*, Sussenbach and Strijkert (1969) found that ornithine transcarbamylase is a constitutive enzyme. The other enzymes of the pathway are not regulated to any extent except the terminal enzyme of the arginine pathway, namely argininosuccinate lyase, which exhibits strong repression and derepression. On the other hand, in *Proteus mirabilis*, Prozesky (1969) reported a different situation. The presence of arginine in the growth medium in this case had surprisingly little effect on the synthesis of arginine enzymes. The small variations in enzyme levels obtained during repression and derepression and the absence of N-acetyl-glutamic- $\gamma$ -semialdehyde dehydrogenase activity led Prozesky (1969) to conclude that the control of arginine enzymes in *Proteus* is unlike the coordination behavior as reported by Glansdorff and Sang (1965) in their work with *E. coli* K-12.

Besides arginase, another catabolic enzyme in the urea cycle, ornithine transaminase, was also detected in cell-free extracts of *Saccharomyces* (Table XII). Ornithine is cleaved to yield L-glutamic- $\gamma$ -semialdehyde which is then converted into  $\Delta'$ -pyrroline-5-carboxylate non-enzymatically. The  $\Delta'$ -pyrroline-5-carboxylate might be converted to glutamate by



dehydrogenation (Middelhoven, 1964), and re-enter the ornithine cycle via acetylation. Like arginase, ornithine transaminase was repressed by urea and induced by arginine (Table XII). The data obtained shows that these two catabolic enzymes are regulated co-ordinately.

*Assimilation of Arginine and Urea by *Saccharomyces cerevisiae*.*

The results summarized in Table XV show that no detectable excretion of amino acids into the culture medium occurred when the cells were incubated in a nitrogen free-medium for 5 hours. The uptake of arginine by yeast cells amounted to 30% of that supplied when this amino acid was the sole source of nitrogen. A considerable quantity (430  $\mu$ moles) of ornithine was detected in the medium, indicating that movement of this amino acid into the medium had occurred. Ammonia was only detected in trace amounts in the medium after this period of growth (Table XIII). These findings suggested that a rapid degradation of arginine into ornithine and urea occurs during this period. In the case of culture in urea-rich media (Table XIII), 25% of the urea supplied was assimilated after 5 hours of growth. At the same time, a total of 1600  $\mu$ moles of ammonia were excreted to the medium while ornithine was only detected in trace amounts. Under these experimental conditions, no other amino acids were detectable in the culture medium. A 30% increase in growth was observed in cells utilizing arginine as the sole nitrogen source, while up to 90% increase in growth resulted when the cells were grown on urea (Table XIV). Both arginine and urea



can clearly serve as sole nitrogen sources for this organism.

When the endogenous pools of amino acids are compared further differences are apparent. When grown on nitrogen free medium, the levels of all amino acids were generally very low (Table XIV). Ammonium and glutamate were by far the principal components found. No free citrulline, ornithine or urea were observed in extracts of the cells and arginine was only found in trace amounts. This suggests that a rapid recycling of the ornithine cycle intermediates is taking place. When arginine was supplied exogenously, increases in the endogenous pool of arginine were observed. The accompanying high levels of urea, suggests an involvement of arginase. In agreement with the results of earlier enzyme experiments, this compound is probably further converted into ammonia as this latter compound was detected in the cell-free extracts (Table XIV). The considerable increase in glutamate under these conditions might be chiefly attributed to utilization of the ammonia produced from urea. The substantial accumulation of ornithine in the medium (Table XIII) can also be related to the induced arginase activity (Table III and IV) of such cells. These accumulations of ornithine would be further amplified by repression of ornithine transcarbamylase. The cells may excrete ornithine to the medium as Ramos *et al.* (1970) have reported that this amino acid is toxic and can readily inhibit protein and nucleic acid biosynthesis in yeast. Considering the amino acid pools of cells grown in urea-rich media (Table XIV) the following changes are



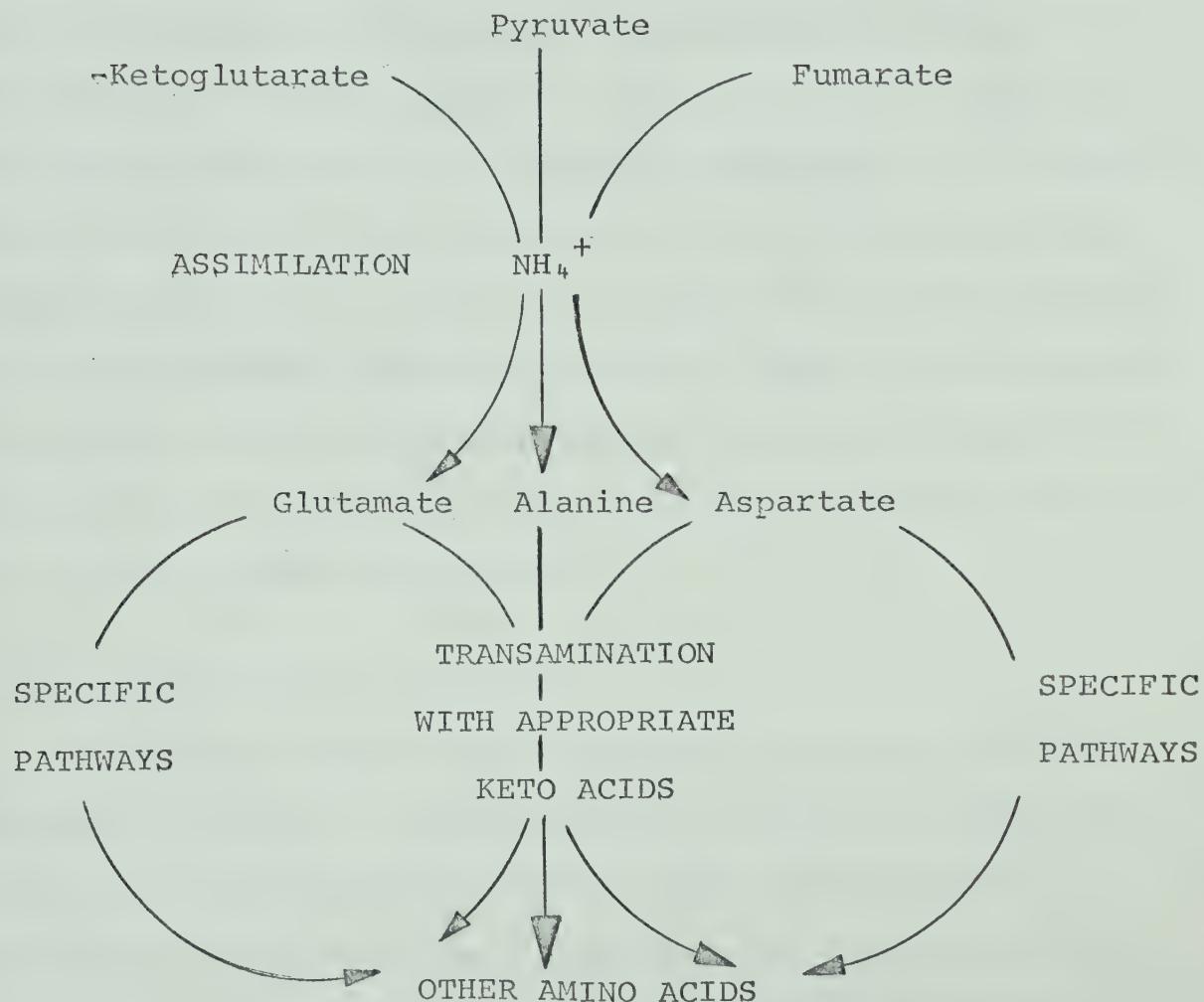
noteworthy. The high level of ammonia (Table XIV) after 5 hours growth suggests that urea was actively degraded into ammonia. As no urease activity was detected in such cells it appears that this ammonia may be formed by urea lyase. In this connection, a partially purified enzyme, from urea-grown *Candida utilis* cells (Roon and Levenberg, 1968) was found to catalyze a  $Mg^{++}$  and  $K^+$  dependent decomposition of urea in 1 mole of carbon dioxide and 2 moles of ammonia. This was accompanied by cleavage of a mole of ATP inorganic phosphate. Direct assays of this activity in *Saccharomyces cerevisiae* were also detected (Whitney and Cooper, 1970), indicating that this reaction may be of importance particularly when urea is the sole source of nitrogen.

Yeast cells are, apparently, capable of synthesizing all of the amino acids required for their growth from inorganic nitrogen, in the form of ammonia, and from carbon skeletons derived from carbohydrate catabolism (Moat and Ahmad, 1965). This is supported in the present studies, by the observed increases in the amino acids in cells grown in urea-rich media (Table XIV). A pool of urea was not found in such cells, suggesting that this compound was actively assimilated, being splitted into ammonia and presumably into carbon dioxide. Despite the rise in the amino acid pools, the pool of ammonia, in all cases, was lower than that of most amino acids. About 20% of this ammonia was assimilated, the remaining 80% being excreted to the medium (Table XIII). Ammonia is known to be assimilated by yeast cells (Figure 17). This utilization is directly related to synthesis of several key amino acids



FIGURE 17

GENERAL PATHWAYS OF AMMONIA ASSIMILATION AND  
AMINO ACID BIOSYNTHESIS





(Moat and Ahmad, 1965). Such amino acids can then serve as donors of amino nitrogen to form many of the other amino acids via transamination reactions.

With the exception of arginine other intermediates of the Krebs-Henseleit cycle were not detected in the present amino acid analyses. A possible explanation for this, particularly following culture in the presence of urea, is that arginase activity was repressed resulting in an accumulation of arginine. This accumulation would then exert both feedback inhibition on the first enzyme of the cycle, namely N- $\alpha$ -acetylglutamate reductase (DeDeken, 1962), and repression of ornithine transcarbamylase (Bechet and Wiame, 1965). Consequently, the net synthesis of arginine will be curtailed under these culture conditions.

#### *Physical Properties of Arginase*

Yeast arginase has many properties in common with the arginases of animals, plants and other microorganisms. For example, the yeast enzyme catalyzes the conversion of L-arginine to ornithine and urea with a pH optimum of 9.2. In common with the liver enzyme (Van Slyke and Archibald, 1946) a linear relationship between enzyme activity and time was maintained for only 10 minutes. This short time relationship (Figure 10) can be chiefly ascribed to the strong inhibitory effect displayed by ornithine (Figure 14). The partially purified yeast arginase was activated by manganese, cobalt and ferrous ions (Table XV) the former ion being the most effective. A comparison of the  $K_m$  value for



arginine (12 mM) reveals a similarity between the yeast enzyme and those of rat and bovine liver (10-20 mM). Mora *et al.* (1965) reported high  $K_m$  values for L-arginine in studies of the enzyme from chicken liver and *Neurospora*. The values ranged between 100 and 200 mM. In the present study the  $K_m$  for arginine was found to vary when different buffer systems were used. The results in Table XVI show that with sodium glycinate as the buffer, the  $K_m$  values for L-arginine averaged 12.5 mM. This value was raised to 250 mM when potassium phosphate was used as buffer. As Middelhoven (1969) has concluded that phosphate is an inhibitor of the native enzyme the present change in the  $K_m$  for arginine suggests that phosphate may exert its effect on the arginine binding site.

When the effects of urea and L-ornithine on arginase were studied *in vitro*, it was found that urea had no influence on the activity while L-ornithine was highly inhibitory (Tables IX, X and XI). The removal of such inhibition following gel filtration suggests that the binding of ornithine to the enzyme is readily reversible. Kinetic studies of this inhibition clearly indicated that it is competitive in nature (Figure 14).

The molecular weight of yeast arginase was found to be approximately 120,000 ( $\pm$  10,000) as determined by Sephadex G-200 gel filtration (Figure 16). At the same time, the arginase was purified by this treatment, the specific activity being increased 30 fold. On the basis of these studies, it can be concluded that the yeast enzyme belongs to the 120,000



molecular weight class which includes the enzymes from bovine liver (M. Wt. 138,000, Greenberg *et al.*, 1956) and rat liver, (M. Wt. 134,000, Mora *et al.*, 1966). More recently, Hirsch-Kolb and Greenberg (1968) have found the molecular weight of rat liver arginase to be 118,000, furthermore, the enzyme is composed of our subunits, each of 30,800 molecular weight. It is of interest to note that this subunit has approximately the same molecular weight as the earthworm arginase (M. Wt. 27,000) reported by Reddy and Campbell (1968). To date, this is the lowest molecular weight known for a molecule with arginase activity. It is significant that an active arginase unit of this small dimension exists since the subunit structure of arginase is unknown. The other major group of arginases belongs to a 240,000 molecular weight class which includes the enzymes from chicken liver (Mora *et al.*, 1966), insect fatty body (Reddy and Campbell, 1969), and *Neurospora* (Mora *et al.*, 1964). In their studies with insect fatty body arginase, Reddy and Campbell (1969) obtained an equilibrium mixture containing proteins of 240,000 and 120,000 molecular weights, indicating that such arginases may undergo extensive association and dissociation. If the earthworm arginase is, in fact, a naturally occurring monomer, the arginases of other species could be associations of this monomer to form octamers (8 x 30,000), tetramers 4 x 30,000) or molecules of intermediary size.

Table XVII summarizes the physical properties of





TABLE XVII

\* - unknown;

\*\* - based on the work of Mora *et al.* (1966);

\*\*\* - based on the work of Carlisky *et al.* (1967);

- PCMB: abbreviation for p-chloromercuribenzoate;

c - competitive;

n-c - non-competitive

Based on data from:

<sup>1</sup> Mohamed and Greenberg (1945); Greenberg *et al.* (1956);

<sup>2</sup> Mora *et al.* (1965 and 1966);

<sup>3</sup> Carlisky *et al.* (1967)

<sup>4</sup> Reddy and Campbell (1969)

<sup>5</sup> Muszynska and Reifer (1968)

<sup>6</sup> this study

<sup>7</sup> Bishop and Campbell (1965); Reddy and Campbell (1968).

TABLE XVII. SUMMARY OF THE PROPERTIES OF ARGINASES FROM VARIOUS SOURCES

Species	$K_m$ for L-arginine (mM)	pH Optimum	Activators	Inhibitors	Inhibition by L-ornithine	Molecular Weight
Bovine liver <sup>1</sup>	6 - 7	9.2	Mn <sup>++</sup> , Ni <sup>++</sup> , Co <sup>++</sup>	Hg <sup>++</sup> , Ag <sup>++</sup> , Zn <sup>++</sup>	--*	138,000
Rat liver <sup>2</sup>	20 - 40	--	Mn <sup>++</sup> , Ni <sup>++</sup>	--	n-c	134,000
Chicken liver <sup>2</sup>	100 - 200	--	Mn <sup>++</sup>	PCMB†	c	276,000
Frog kidney <sup>3</sup>	20 - 40	9.6-9.7	Mn <sup>++</sup>	--	c	--
Silkworm fatty body <sup>4</sup>	2 - 4	9.8	Mn <sup>++</sup> , Co <sup>++</sup> , Ni <sup>++</sup> , Cd <sup>++</sup>	--	c	228,000
Bitter lupin <sup>5</sup>	30 - 40	10.4	Mn <sup>++</sup> , Co <sup>++</sup> , Ni <sup>++</sup>	chlorogenic acid	--	--
Neurospora <sup>2, 3</sup>	100 - 200	9.5	Mn <sup>++</sup>	PCMP	c** n-c***	278,000
Yeast <sup>6</sup>	10 - 20	9.20	Mn <sup>++</sup> , Co <sup>++</sup> , Fe <sup>++</sup>	Na <sub>2</sub> S, Ag <sup>++</sup> pot. phosphate	c	120,000
Earthworm gut <sup>7</sup>		9.5	Mn <sup>++</sup>	--	--	29,500



arginases from various sources including animals, plants and microorganisms. Mora *et al.* (1966) reported that the arginase of *Neurospora crassa* is of the type found in uricotelic animals with respect to the  $K_m$  for arginine, the inhibition by ornithine and a number of other characteristics. In the present research, the physical characteristics of yeast arginase are similar to those of the mammalian enzyme, the only exception being the type of ornithine inhibition. Such inhibition was found to be purely competitive in the yeast enzyme (Figures 14, 15) but in the case of the other enzymes is both competitive and non-competitive (Mora *et al.*, 1966). On the other hand, considering the arginine  $K_m$ , inhibition by ornithine and molecular weight, it can be concluded that the yeast enzyme is distinct from that of *Neurospora*.

As mentioned in the Introduction, in their work on the synthesis of ornithine transcarbamylase in *Saccharomyces cerevisiae*, Messenguy and Wiame (1969) were able to isolate a regulatory protein which bound with the OTC enzyme in the presence of arginine and ornithine. This regulatory protein was subsequently proved to be an arginase which displayed a different chromatographic behavior when bound with OTC, hence was distinguishable following chromatography. These workers suggested that this type of regulation may be general in the genus *Saccharomyces* but, at the same time, may not be common in other species.

In the present work, the writer attempted to examine the possible existence of a regulatory protein which may be



involved in control of the catabolic pathway of the Krebs-Henseleit cycle. Chromatography using Sephadex G-200 gave similar elutions for arginase extracted from cells grown in arginine-rich and urea-rich media (Figure 12). Clearly, repression of arginase by urea was not associated with an increase in molecular weight as would be expected if a regulatory protein was involved.

#### *Concluding Remarks*

To summarize the present and related work on the regulation of the Krebs-Henseleit cycle in *S. cerevisiae*, the reader may refer to Figure 18.

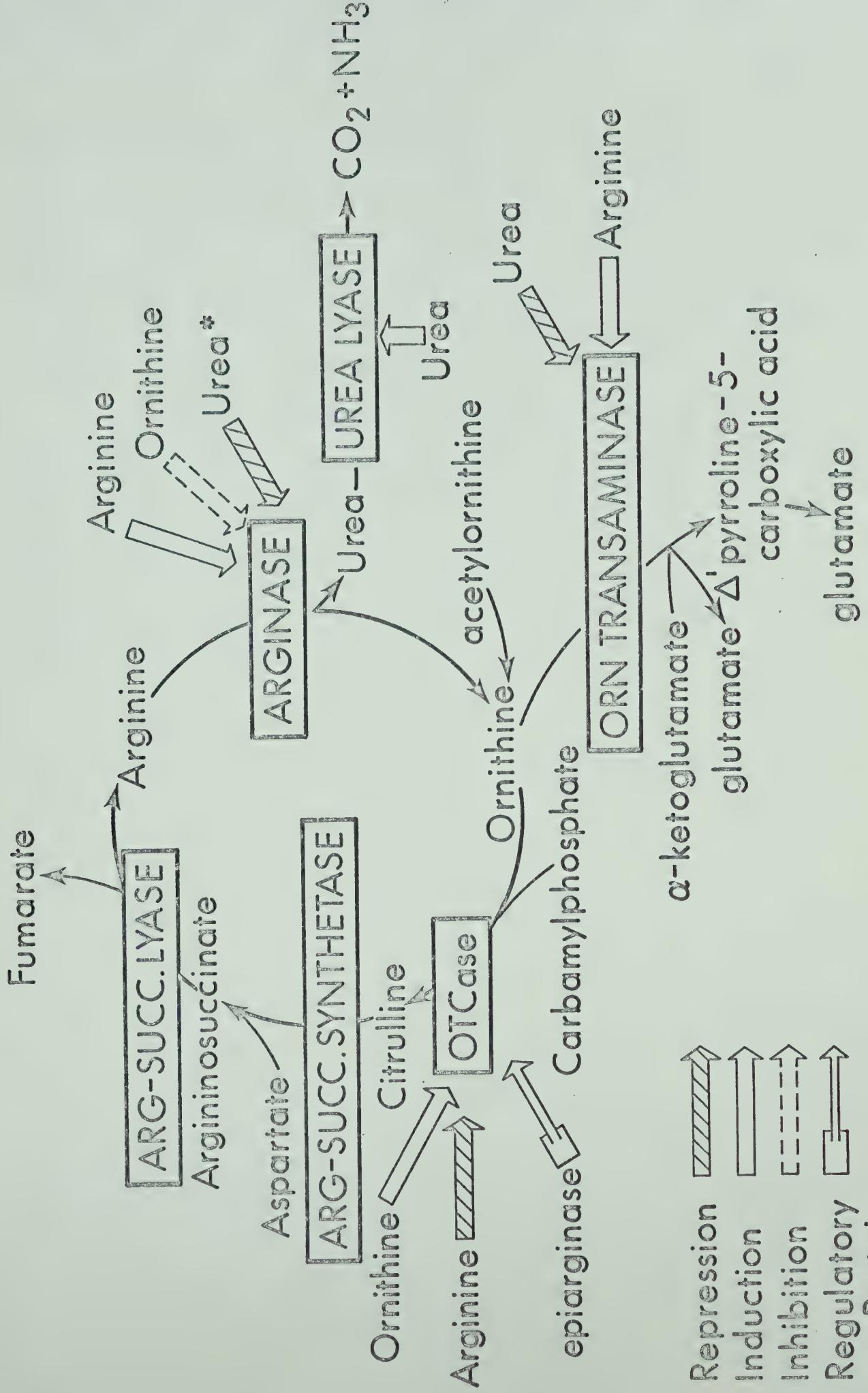
Many investigations have shed light on the mechanisms for regulation of this important cycle. However, some controversy still exists, particularly with regard to the relative importance of glutamate, aspartate, and alanine in the utilization of urea and ammonia. Furthermore, the sources of nitrogen and/or carbon in the formation and interconversion of other amino acids and other nitrogenous compounds in the yeast cells remain to be fully elucidated. Little is known about the genetic basis for regulation of arginase and ornithine transaminase. In this connection, it is of importance to determine whether urea serves truly as a co-repressor in exerting its effect on the formation of arginase. Alternatively this effect may be expressed indirectly through transfer RNA which may be the real co-repressor, a situation confirmed in many regulatory pathways (Anton, 1968; Eidelic and Neidhardt, 1965) including that of





FIGURE 18

*POSSIBLE MECHANISMS FOR REGULATION OF THE KREBS-  
HENSELEIT CYCLE AND ASSOCIATED PATHWAYS  
IN *SACCHAROMYCES CEREVISIAE**



\* Smaller amounts of repression occur with ornithine



arginine metabolism in yeast (Ramos *et al.*, 1970). The exact mechanism for urea utilization in the absence of urease is also not clear. The metabolic interrelationships of glutamate, ornithine and proline also deserve further study. Future studies should also emphasize the energetics of urea and ammonia utilization in this organism.

Finally, although the general features of the Jacob and Monod model for cellular control are plausible, many of the details of regulation remain to be clarified. Perhaps one must be aware that in such studies the details of regulation in one model may not be universally applicable to all other living systems.



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